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CAFEÍNA E ESTRESSE: INFLUÊNCIAS SOBRE O COMPORTAMENTO E SOBRE PARÂMETROS BIOQUÍMICOS AVALIANDO ESTRESSE OXIDATIVO NO SISTEMA NERVOSO CENTRAL

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do Grau de Mestre em Ciências Biológicas: Bioquímica.

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DEDICATÓRIA

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SUMÁRIO

RESUMO

A cafeína é uma substância amplamente consumida na forma de café, chás e refrigerantes. Estudos demonstram sua influência no comportamento alimentar aparentemente promovendo uma pequena redução na ingestão de calorias e no comportamento do tipo ansioso, onde altas doses podem induzir um estado de ansiedade. Além disso, ela parece ser neuroprotetora, e é possível que parte desse efeito seja devido a sua atividade antioxidante a qual está bem descrita *in vitro*. O consumo de cafeína muitas vezes está associado a situações de estresse. A exposição ao estresse crônico leva a alterações bioquímicas e comportamentais, tendo sido sugerido que aumenta a produção de radicais livres. O objetivo deste trabalho é verificar o efeito do consumo de cafeína e do estresse crônico sobre os parâmetros comportamentais, comportamento do tipo ansioso e alimentar e sobre parâmetros bioquímicos relacionados ao estresse oxidativo em hipocampo, estriado e córtex cerebral de ratos. Além disso, diferenças sexo-específicas foram verificadas com relação ao comportamento do tipo ansioso e ao dano ao ADN. Ratos Wistar machos e fêmeas foram submetidos ao estresse por contenção por 40dias. A dieta consistiu de ração padrão e cafeína 0,3 ou 1g/L na água de beber, a vontade, como única fonte de líquido disponível. Controles receberam água da torneira. A ansiedade e o comportamento alimentar foram avaliados, bem como o dano ao ADN (em hipocampo), a lipoperoxidação, o Potencial antioxidante reativo total (TRAP) e a atividade das enzimas antioxidantes Superóxido dismutase (SOD), Glutationa peroxidase (GPx) e Catalase (CAT), no hipocampo, estriado e córtex cerebral. O consumo de cafeína e o estresse crônico aumentaram o comportamento do tipo ansioso, efeito que foi observado apenas nos machos. No comportamento alimentar, a cafeína (0,3g/L e 1g/L) aumentou a latência e diminuiu o consumo de alimento doce (Froot Loops®) no estado alimentado. Além disso, a cafeína 1g/L levou a um consumo menos intenso de alimento salgado (Cheetos®) pelos ratos no estado de jejum. Tanto a cafeína quanto o estresse aumentaram o dano ao ADN no hipocampo de ratos machos sem haver alteração nas fêmeas. Com relação ao estresse oxidativo, houve interações entre estresse e cafeína, especialmente no córtex, na atividade da SOD e da CAT, uma vez que a cafeína aumentou a atividade destas enzimas nos animais controles e não teve efeito nos estressados Além disso, o estresse diminuiu a atividade da GPx no córtex e aumentou a atividade da SOD no estriado. Conclui-se que o estresse crônico e o consumo de cafeína levaram a um aumento no comportamento do tipo ansioso em ratos machos. Além disso, a cafeína aumentou a latência e diminuiu o consumo de Froot Loops® em ratos previamente alimentados. Por outro lado, o estresse repetido por contenção induziu um estado de maior susceptibilidade ao estresse oxidativo em algumas estruturas cerebrais devido a atividade alterada das enzimas antioxidantes, enquanto que, a administração crônica de cafeína levou em alguns casos (depende da estrutura) a um aumento na atividade de enzimas antioxidantes, sugerindo um papel neuroprotetor, que depende do estado do sujeito (estressado ou não).

ABSTRACT

Caffeine is widely consumed in coffee, tea and soft-drinks. Studies show its influence on feeding behavior apparently through slight reduction on calorie ingestion and on anxiety-like behavior, where high doses can induce anxiety states. Besides, it seems to be neuroprotective, and it is possible that part of this effect is due to its antioxidant activity, which is well described *in vitro*. Caffeine consumption is many times associated to stressfull situations. The exposure to chronic stress leads to biochemical and behavioral changes and it has been suggested that increases free radicals production. The aim of this work is to verify the effect of caffeine consumption and chronic stress on behavioral parameters, anxiety-like-behavior and feeding behavior and on biochemical parameters related to oxidative stress in hippocampus, striatum and brain cortex of rats. Besides, sex specifics differences were verified on anxiety-like behavior and on DNA damage. Wistar rats male and female were submitted to restraint stress for 40 days. The diet was standard chow and caffeine 0.3g/L or 1g/L in the drinking water, *ad libitum*, as the only source of drinking. Controls received tap water. Anxiety-like-behavior and feeding behavior were evaluated as well as DNA damage (in the hippocampus), lipoperoxidation, Total reactive antioxidant potential (TRAP), and antioxidant enzymes ativities, Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) and Catalase (CAT) in the hippocampus, striatum and brain cortex. The caffeine consumption and chronic stress increased anxiety-like behavior, effect which was observed only in males. On feeding behavioral, caffeine 0.3g/L and 1g/L increased the latency and decreased the consumption of sweet food (Froot Loops®) on feeding state. Besides, caffeine 1g/L led to a less intense consumption of salty food (Cheetos®), on fasting state. Both caffeine and stress increased the DNA damage in the hippocampus of male rats, without any alteration on females. About oxidative stress, there were interactions between caffeine and stress, specially on cortex, on SOD and CAT activities, once caffeine increased the activity of these enzymes in control animals and had no effect in stressed animals. Furthermore, stress decreased GPx activity in the cortex and increased SOD activity in the estriatum. In conclusion, chronic stress and caffeine consumption led to an increase on anxiety-like behavior in male rats. Besides, caffeine increased the latency and decreased the consumption of Froot Loops® in pre-feeding rats. On the other hand, repeated restraint stress induced a state of higher susceptibility to oxidative stress in some structures, considering the altered activities of antioxidant enzymes, while the chronic administration of caffeine, led, in some cases (depending on the structure), to increased activity of antioxidant enzymes, suggesting a possible neuroprotective role, which depends on the subject state (stressed or not).

LISTA DE ABREVIATURAS

ACTH = Hormônio adrenocorticotrófico

ADN = Ácido desoxirribonucleico

CAT = Catalase

CGS15943A = 9-cloro-2-(2-furanil)-5,6-dihidro-[1,2,4]triazolo[1,5]quinazolin-5-imine

CPX = 3-di-propil-8-ciclopentilxantina

EROs = Espécies reativas do oxigênio

GPx = Glutationa peroxidase

SOD = Superóxido dismutase

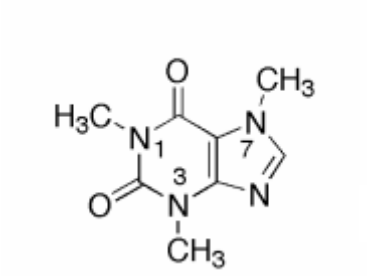
TBARS = Espécies reativas ao ácido tiobarbitúrico

TRAP = Potencial antioxidante reativo total

1. INTRODUÇÃO

1.1 Cafeína

A cafeína é uma xantina designada quimicamente como 1,3,7-trimetilxantina (Figura 1).



CAFEÍNA

Figura 1: Estrutura química da cafeína. Fonte: DALY (2007)

É a substância psicoativa mais consumida no mundo, na forma de bebidas como café, chás, refrigerantes a base de cola e alimentos como o chocolate. Muitas pessoas acreditam que o componente ativo do chá é a teofilina. No entanto, a cafeína, e não a teofilina, é a principal xantina encontrada no chá, apesar de a cafeína ser metabolizada em teofilina *in vivo* (HIRSH, 1984). Os níveis de cafeína nos alimentos variam significativamente (ver Tabela 1), sendo que o café tipicamente contém mais cafeína, e o chá contém quantidades intermediárias (LIEBERMAN, 2003).

Tabela 1: Conteúdo de cafeína em produtos alimentícios

Produto	Volume ou Peso	Conteúdo médio de cafeína (mg)
Café moído e torrado	150ml	83
Café instantâneo	150ml	59
Chá em saquinhos	150ml	30
Chá em folhas	150ml	41
Chá instantâneo	150ml	28
Chocolate em barra	28g	20
Chocolate ao leite	28g	6
Achocolatado	240ml	5
Refrigerante de cola	180ml	19
Refrigerante de cola diet	180ml	21

Fonte: Adaptado de BARONE e ROBERTS, 1996.

O consumo agudo e especialmente o consumo crônico de cafeína parecem ter apenas pequenas conseqüências negativas na saúde. Por esta razão, e porque poucos usuários de cafeína relatam perda do controle sobre o consumo desta substância, as agências governamentais não impuseram restrições no consumo de cafeína (FREDHOLM ET AL., 1999). No entanto, há alguns anos atrás, foi descrito que a cafeína pode ser uma potencial droga de abuso (GILLILAND e BULLOCK, 1984). Entre as ações da cafeína está a sua capacidade de bloquear os efeitos da adenosina nos receptores A1 e A2A, inclusive nas baixas concentrações alcançadas após o consumo de uma xícara de café. Outra das possíveis ações da cafeína seria a inibição da quebra de nucleotídeos cíclicos, via inibição da fosfodiesterase; no entanto, seria necessária uma concentração 20 vezes maior para alcançar esse efeito; para bloquear os receptores GABAA, a concentração deve ser 40 vezes maior; e para mobilizar o cálcio dos

depósitos intracelulares são necessárias concentrações 100 vezes maiores (FREDHOLM ET AL., 1999).

1.1.1 Cafeína e Comportamento

A cafeína apresenta diversas influências no comportamento, entre elas o aumento no estado de alerta, motivação para trabalhar e eficiência (FREDHOLM ET AL., 1999). Os efeitos da cafeína no aprendizado, na memória e na coordenação estão relacionados à ação dessa metilxantina no alerta, na vigilância e na fadiga (NEHLIG, DAVAL e DEBRY, 1992). O tratamento crônico com cafeína causa uma diminuição na atividade locomotora (NIKODIJEVIC, JACOBSON e DALY, 1993), enquanto que o tratamento agudo com moderadas doses, estimula a atividade locomotora em roedores (FREDHOLM ET AL., 1999), mas altas doses também diminuem essa atividade.

A cafeína no comportamento alimentar parece ter um pequeno efeito redutor da ingestão calórica (TREMBLAY ET AL., 1988), o qual é similar, apesar de menos intenso, ao observado pelas anfetaminas (FOLTIN, KELLY e FISCHMAN, 1995). Para ambas as drogas o efeito ocorre no número de refeições consumidas ao invés de no tamanho da refeição (FREDHOLM ET AL., 1999).

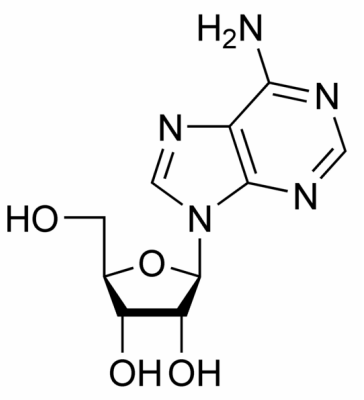
Outro aspecto comportamental influenciado pela cafeína é a ansiedade. A administração de doses altas de cafeína leva a um aumento da ansiedade em humanos, o que não é observado em baixas doses (BOULENGER ET AL., 1987). Similarmente, a administração aguda de altas doses de cafeína promove comportamento do tipo ansioso em diferentes modelos animais de ansiedade, como o teste de interação social (BALDWIN e FILE, 1989) ou o teste do labirinto em cruz elevado (EL YACOUBI ET AL., 2000).

Conforme descrito acima, a cafeína é um antagonista não seletivo de receptores de adenosina A1 e A2. Foi demonstrado que o antagonista seletivo do receptor A1, 1,3-di-propil-8-ciclopentilxantina (CPX) não apresenta efeito no comportamento do tipo ansioso, enquanto que o antagonista de receptores A1 e A2, 9-cloro-2-(2-furanil)-5,6-diidro-[1,2,4]triazolo[1,5]quinazolin-5-imina (CGS15943A) apresenta efeitos ansiogênicos (GRIEBEL ET AL., 1991). Sugere-se ainda que mecanismos adaptativos seguidos de alterações nos receptores A2A ou seu bloqueio após a ingestão crônica de cafeína podem ser responsáveis pelo aumento na suscetibilidade à ansiedade (EL YACOUBI ET AL., 2000). No entanto, ainda não está completamente esclarecido o papel destes receptores no comportamento do tipo ansioso (CORREA e FONT, 2008).

1.1.2 Adenosina, Cafeína e Estresse Oxidativo

A adenosina está presente em todos os tecidos do organismo de mamíferos, modulando vários processos fisiológicos importantes (WARDAS, 2002). Vários trabalhos indicam que a adenosina pode ser um agente neuroprotetor endógeno no Sistema Nervoso Central, uma vez que ela previne o dano causado por isquemia e excitotoxicidade. Várias evidências também apoiam o papel dos receptores de adenosina A1 e A2A nos mecanismos neuroprotetores. Por exemplo, a cafeína protege contra neurotoxicidade em modelos animais de Doença de Parkinson (KALDA, 2006; XU, 2006) e, em humanos, tem sido observada uma relação inversa entre o consumo de cafeína e a prevalência de desenvolver Doenças de Parkinson ou Alzheimer (ROSS, 2000; MAIA e DE MENDONÇA, 2002). Estudos demonstram que a estimulação aguda dos receptores A1 ou bloqueio de receptores A2A conferem um similar efeito neuroprotetor contra vários estímulos nocivos cerebrais envolvendo estresse oxidativo

(DE MENDONÇA, SEBASTIÃO e RIBEIRO, 2000; RUDOLPHI, SCHUBERT e PARKINSON, 1992; VON LUBITZ, 1999). Devemos lembrar também que estes receptores sofrem regulação e, portanto, em relação à neuroproteção resultados opostos podem ser obtidos quando esses receptores são ativados ou bloqueados cronicamente. Além disso, a administração aguda de adenosina aumenta a atividade de enzimas antioxidantes e foi demonstrado que a expressão de receptores de adenosina A1 é regulada pelo estresse oxidativo, o qual aumenta a expressão pela ativação do fator nuclear *k*B (NIE ET AL., 1998). Por outro lado, o estresse oxidativo diminui a expressão de receptores de adenosina A2A (RAMKUMAR, HALLAM e NIE, 2001).



ADENOSINA

Figura 2: Estrutura química da adenosina.

Fonte:WIKIPEDIA <http://pt.wikipedia.org/wiki/Ficheiro:A_chemical_structure.png>

A cafeína age antagonizando os receptores de adenosina A1 e A2A. Isso leva ao seguinte questionamento: a cafeína, quando utilizada cronicamente, apresenta ação neuroprotetora ou neurotóxica?

Alguns estudos, *in vitro*, demonstram que a cafeína e seus metabólitos apresentam efeitos antioxidantes (GÓMEZ-RUIZ, LEAKE e AMES, 2007; LEE, 2000; SHI, DALAL e JAIN, 1991). A cafeína inclusive é capaz de reagir com o radical hidroxila, o qual é gerado pela reação de Fenton (SHI, DALAL e JAIN, 1991), na qual o

peróxido de hidrogênio reage com o ferro e também na reação de Haber Weiss onde o peróxido de hidrogênio reage com o ânion superóxido (HABER e WEISS, 1934). A maioria dos estudos sobre cafeína e estresse oxidativo foram feitos *in vitro,* mas estudos feitos *in vivo* sugerem que a cafeína pode aumentar a atividade de enzimas antioxidantes (MUKHOPADHYAY, MONDAL e PODDAR, 2003; ROSSOWSKA e NAKAMOTO, 1994). Por outro lado, a ação neurotóxica desta substância não pode ser descartada. Foi demonstrado que a administração intraperitoneal de cafeína (50mg/Kg, 3 vezes ao dia) produz morte neuronal em várias regiões do cérebro de ratos neonatais, no entanto esse efeito não foi mediado pelo estresse oxidativo (KANG, LEE e WON, 2002). Estudos também mostram que a cafeína e os produtos do seu catabolismo, teobromina e xantina exibem tanto propriedades antioxidantes (GÓMEZ-RUIZ, LEAKE e AMES, 2007; AZAM ET AL., 2003) quanto pro-oxidantes, demonstrada pela quebra oxidativa do ADN *in vitro* (AZAM ET AL., 2003).

1.2 Estresse

A resposta ao estresse leva a alterações comportamentais e metabólicas, num esforço de manter a homeostasia corporal e aumentar as chances de sobrevivência (CHROUSOS e GOLD, 1992; TSIGOS e CHROUSOS, 2002). Um dos principais mecanismos endócrinos é a ativação do eixo hipotalálamo-hipófise-adrenal, (AGUILERA, 1994), onde inicialmente ocorre a liberação do fator liberador de corticotrofina do hipotálamo, que leva à liberação do hormônio adrenocorticotrófico (ACTH) da hipófise para a circulação sangüínea. O ACTH por sua vez, estimula a

liberação de glicocorticóides (cortisol em humanos e corticosterona em ratos) do córtex da adrenal (LUPIEN ET AL., 2005).

A exposição ao estresse por contenção induz a liberação de glicocorticóides (TSIGOS e CHROUSOS, 2002) e a exposição repetida a altos níveis desses hormônios leva a uma *down-regulation* dos receptores de glicocorticóides no hipocampo, o que prejudica a capacidade deste em controlar a retroalimentação negativa de glicocorticóides (SAPOLSKY, KREY e MCEWEN, 1984). Isso leva a uma hipersecreção posterior de glicocorticóides que acredita-se produzir alterações neuronais em várias regiões cerebrais, incluindo o hipocampo (SAPOLSKY, KREY e MCEWEN, 1984). Além disso, os glicocorticóides têm vários efeitos no organismo, como o aumento da disponibilidade de substrato energético em diferentes partes do corpo e a adaptação às alterações do ambiente (LUPIEN ET AL., 2005).

Outra alteração observada durante o estresse é o aumento na concentração extracelular de adenosina (SCACCIANOCE ET AL., 1989). O estresse repetido por contenção modifica o equilíbrio entre a densidade de receptores de adenosina A1 e A2A no hipocampo de ratos, promovendo uma *down-regulation* de receptores A1 junto com uma *up-regulation* dos receptores A2A (CUNHA ET AL., 2006). Isto sugere que animais estressados repetidamente podem responder de modo diferente à administração de cafeína.

1.2.1 Estresse e Comportamento

O estresse pode alterar o aprendizado e a memória (OITZL ET AL., 2001), sendo aceito de forma geral que eventos estressantes são muito bem lembrados (OLFF,

LANGELAND e GERSONS, 2005). Estudos com animais demonstraram que o estresse facilita e pode até ser indispensável para o aprendizado e a memória (OITZL ET AL., 2001). Um aumento nos níveis dos hormônios do estresse, principalmente os corticosteróides, dentro do contexto da situação de aprendizado ajudam a lembrar o evento em particular. Por outro lado, o estresse também tem sido associado com um prejuízo no desempenho cognitivo em certas situações (JOELS, PU e WIEGERT, 2006).

Além de influenciar a memória, o estresse também promove alterações no comportamento alimentar. Foi demonstrado que o estresse por contenção aumenta o apetite por alimento doce independentemente da condição alimentar (estado alimentado ou jejum), sem aumentar o consumo habitual de ração. Sugere-se que esta alteração esteja relacionada com um aumento nos níveis de ansiedade, pois foi revertida pela administração de diazepam (ELY ET AL., 1997). Outros estudos demonstraram que a exposição crônica a estressores de determinada gravidade, diminui a ingestão alimentar em ratos e leva a uma hipertrofia adrenal. Dessa forma, o tipo, a duração ou a gravidade do estresse podem provocar diferentes respostas no comportamento alimentar (HARGREAVES, 1990; MARTI, MARTI e ARMARIO, 1994).

Da mesma forma que no comportamento alimentar, o estresse pode induzir ou aliviar o comportamento do tipo ansioso em ratos, dependendo do tipo e da gravidade do estressor (D'AQUILA ET AL., 1994; DUCOTTET e BELZUNG, 2004; VAN GAALEN e STECKLER, 2000). Tem sido demonstrado que o estresse agudo aumenta o comportamento do tipo ansioso (MACNEIL ET AL., 1997; MORILAK ET AL., 2003). Ratos submetidos a uma única sessão de choque inescapável apresentaram um aumento deste comportamento 14 dias depois (VAN DIJKEN ET AL., 1992). Por outro

lado, ratos submetidos ao estresse crônico variado apresentaram um perfil ansiolítico no labirinto em cruz elevado (D'AQUILA ET AL., 1994).

Outra consideração importante a fazer é que existem diferenças sexo-específicas em muitos aspectos metabólicos e comportamentais, e uma significativa quantidade de evidências sugerem possíveis interações entre estresse e sexo na adaptação cerebral, no comportamento e na resposta endócrina (BUJAS ET AL., 1997; KANT ET AL., 1983; TROISI, 2001).

1.2.2 Exposição ao Estresse e Estresse Oxidativo

Como descrito acima, o estresse promove a liberação de glicocorticóides. Existem fortes evidências sugerindo que altos níveis de glicocorticóides no cérebro podem produzir efeitos deletérios como dano a neurônios, o que tem sido associado a um aumento na geração de espécies reativas do oxigênio (EROs) (MCINTOSH e SAPOLSKY, 1996). Além disso, EROs produzidos em resposta ao estresse, isto é, ânion superóxido, radical hidroxila e peróxido de hidrogênio, causam peroxidação lipídica, especialmente nas membranas (KOVÁCS ET AL., 1996), mas também podem danificar as proteínas e o ADN celular (COCHRANE, 1991). Tem sido sugerido que, em humanos, o estresse associado ao trabalho, além de condições de trabalho ambientais e físicas, tem uma forte influência no dano oxidativo do ADN, o qual parece ser sexo específico, predominando em mulheres (IRIE ET AL., 2001).

O sistema nervoso central é especialmente vulnerável aos danos dos radicais livres, devido ao alto consumo de oxigênio pelo cérebro, conteúdo abundante de lipídios e insuficiência relativa de enzimas antioxidantes comparada com outros tecidos (HALLIWELL e GUTTERIDGE, 1985). O dano induzido por EROs nas células é

normalmente combatido por sistemas antioxidantes enzimáticos e não-enzimáticos (HALLIWELL e CROSS, 1994). O sistema antioxidante enzimático é composto por enzimas como a superóxido dismutase (SOD), que converte radical superóxido em peróxido de hidrogênio, a catalase (CAT), que promove a degradação do peróxido de hidrogênio, e a glutationa peroxidase (GPx) que promove a degradação de peróxidos, especialmente os derivados da oxidação dos fosfolipídeos de membrana (KEHRER, 2000). A exposição a situações de estresse parece prejudicar as defesas antioxidantes, levando a dano oxidativo por alterar o equilíbrio entre os fatores antioxidantes e oxidantes (MCINTOSH, CORTOPASSI e SAPOLSKY, 1998).

2. OBJETIVOS

2.1 Objetivo geral

Verificar as interações entre o consumo crônico de cafeína e o estresse crônico em parâmetros comportamentais e em parâmetros relacionados ao estresse oxidativo. Será que o consumo de cafeína modifica os efeitos do estresse crônico?

2.2 Objetivos específicos

- Verificar os efeitos do consumo de cafeína e do estresse crônico sobre os parâmetros comportamentais:

  - comportamento do tipo ansioso (Labirinto em Cruz Elevado e Campo Aberto),

  - atividade motora (Campo Aberto),

  - comportamento alimentar (consumo de alimento – Froot Loops® e Cheetos®);

- Adicionalmente, estudar as diferenças sexo-específicas do consumo de cafeína e do estresse crônico sobre o comportamento do tipo ansioso e o dano ao ADN no hipocampo;

- Avaliar os efeitos do consumo de cafeína em animais cronicamente estressados e controles sobre parâmetros bioquímicos relacionados ao estresse oxidativo, analisando esses parâmetros em hipocampo, estriado e córtex cerebral:

  - Potencial antioxidante reativo total (TRAP),

  - Enzimas antioxidantes (GPx, SOD e CAT),

- Lipoperoxidação (por meio da verificação de Espécies Reativas ao Ácido Tiobarbitúrico – TBARS).

3. MATERIAIS E MÉTODOS E RESULTADOS

Os materiais e métodos e os resultados desta dissertação estão apresentados a seguir, da seguinte forma:

- Capítulo 1: Artigo submetido à revista Pharmacology, Biochemistry and Behavior;

- Capítulo 2: Artigo submetido à revista Neurochemical Research;

- Capítulo 3: Materiais e métodos e resultados adicionais.

3.1 Capítulo 1

Sex-specific differences on caffeine consumption and chronic stress induced anxiety-like behavior and DNA damage in the hippocampus

Artigo submetido para publicação na revista Pharmacology, Biochemistry and Behavior.

SEX-SPECIFIC DIFFERENCES ON CAFFEINE CONSUMPTION AND CHRONIC STRESS-INDUCED ANXIETY-LIKE BEHAVIOR AND DNA DAMAGE IN THE HIPPOCAMPUS

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Abstract

Caffeine is widely consumed in beverages and food. Consumption in high doses is associated with anxiety increase. Stress is part of life and often it can be associated to coffee consumption. Besides, work-associated stress, other than physical and environmental conditions, has a strong influence on oxidative DNA damage, which appears to be sex-specific. In addition, caffeine can induce inhibition of the post-replication repair process in mammalian cells. As there are sex specific differences in many metabolic, neurochemical and behavioral aspects, the aim of this study is to verify the interaction between chronic consumption of caffeine and chronic stress on anxiety and DNA damage in the hippocampus on rats. Wistar rats were submitted to restraint stress for 40 days. The diet consisted of standard rat chow and caffeine (0.3 or 1g/L) in drinking water “ad libittum” as the only drinking source. Controls received tap water. Anxiety like behavior and DNA damage in the hippocampus were evaluated. Caffeine consumption and chronic stress increased anxiety like behavior as well as DNA damage in the hippocampus of male rats. No effect on these parameters was observed in females. Therefore, sex differences favor females, and these results may be related to the presence of gonadal hormones.

Keywords: anxiety, caffeine, stress, DNA damage.

Introduction

Caffeine is an ingredient widely used in beverages and foods, including coffee, tea, many soft drinks, and chocolate (MacKenzie et al., 2007). Coffee is the main source of caffeine and its ingestion varies over the day (Brice and Smith, 2002). The primary action of caffeine in moderate doses is believed to be the blockage of adenosine receptors (A1 and A2a), which leads to very important secondary effects on many classes of neurotransmitters like glutamate, acetylcholine and dopamine (Sichardt and Nieber, 2007). However, in high concentrations (at mM range) it may inhibit phosphodiesterase, mobilizes intracellular calcium (Fredholm et al., 1999) and also presents antioxidant activity (Lee, 2000; Devasagayam et al., 1996). This, in turn, it will influence a large number of different physiological functions (Fredholm et al., 1999). In humans the administration of high doses of caffeine leads to an increase in anxiety levels (Clementz and Dailey, 1988). Similarly, an acute administration of high doses of caffeine promotes anxious behaviour in different animal models, such as the social interaction test (Baldwin and File, 1989) or the elevated plus maze test (El Yacoubi et al., 2000). It has also been proposed that differences in the activity of the adenosinergic system are accompanied by differences in anxiety level (Johansson et al., 2001; Ledent et al., 1997). Besides, caffeine and its catabolic products theobromine and xanthine exhibit both antioxidant (Gómez-Ruiz et al., 2007; Azam et al., 2003) and prooxidant properties, showing oxidative DNA breakage “in vitro” (Azam et al., 2003). However, little is known about the effect of chronic caffeine consumption on DNA damage in the central nervous system.

The stress response leads to behavioral and metabolic changes in an effort to maintain body homeostasis and increase survival chances (Chrousos and Gold, 1992; Tsigos and Chrousos, 2002). Exposure to stress induces the release of glucocorticoids (Tsigos and Chrousos, 2002), and repeated exposure to high levels of glucocorticoids produces neuronal changes in several brain regions, including the hippocampus (Sapolsky et al., 1984; Sapolsky et al., 1985). On the other hand, there is an increase in adenosine extracellular concentration during stress (Scaccianoce et al., 1989) and the balance between the density of adenosine A1 and A2A receptors in the rat hippocampus is modified upon repeated immobilization stress, which leads to a down-regulation of A1 receptors together with an up-regulation of A2A receptors (Cunha et al., 2006). Concerning emotional behavior, there is some evidence suggesting that repeated restraint stress produces changes in emotionality related to increased anxiety (Beck and Luine, 2002).

Several studies have examined the effect of stress on DNA integrity, as stress has been found to cause production of reactive oxygen species (ROS) resulting in oxidative stress and increased lipid peroxidation; oxidative stress-induced DNA alterations may affect replication and transcription (Perchellet and Perchellet, 1989). Besides, psychological stress has been shown to impair the repair of DNA damage induced by exposure to a carcinogen in rats (Irie et al., 2000). Additionally, it has been suggested that, in humans, work-associated stress, other than physical and environmental working conditions, has a strong influence on oxidative DNA damage, which appears to be sex-specific (particularly in female workers) (Irie et al., 2001).

Most studies evaluating the effects of the stress were conducted in males, but the response and adaptation to stress and chronic caffeine consumption can be different on females. There are sex specific differences in many metabolic aspects as well as in

behavior and a significant body of evidence implicates interactions between stress and sex on brain adaptation, besides behavior and endocrine responses (Bujas et al., 1997; Kant et al., 1983; Troisi, 2001).

The first aim of the present study is to verify the interaction between chronic consumption of caffeine and chronic stress on anxiety like behavior. The hypothesis is that chronic consumption of caffeine in high doses, associated with chronic stress would increase anxiety like behavior. Since caffeine may alter a post-replication repair process in mammalian cells (Van Den Berg and Roberts, 1976), and as exposure to stress can increase oxidative stress (Perchellet and Perchellet, 1989; Fontella et al., 2005), the second aim of this study is to verify the effect of stress and caffeine on DNA damage in hippocampus, which is a brain structure strongly vulnerable to stress. Additionally, since it was observed that caffeine-induced effects on food consumption are differently affected by stress depending on the sex of the animal (Pettenuzzo et al., 2008), the third aim of this study is to verify possible sex-differences in the effects of these chronic treatments on anxiety like behavior and on DNA damage.

Material and Methods

Animals

For the present study, 58 adult males and 48 adult females Wistar rats from our breeding stock (60 days of age at the beginning of the treatment), weighing 250-300g (males) and 150-200g (females) were used. Animals were separated according to sex on postnatal day 21. Experiments using males and females were performed separately, at different times. Around postnatal day 60, animals from different litters were

randomized, to avoid the litter effect, and maintained in groups from 3-5 animals per cage. One week later, cages were divided between six groups for males and six groups for females. Males and females were kept separated throughout all the experiments. The animals received or not caffeine (0.3 g/L and 1.0 g/L) in drinking water, and were subjected or not to repeated restraint stress during at least 40 days. The final groups were: control (non-stressed receiving water), caffeine 0.3g/L (non-stressed receiving caffeine 0.3g/L), caffeine 1.0g/L (non-stressed receiving caffeine 1.0g/L), stressed (stressed receiving water), stressed + caffeine 0.3g/L (stressed receiving caffeine 0.3g/L) and stressed + caffeine 1.0g/L (stressed receiving caffeine 1.0g/L), resulting in 12 groups, considering males and females. Cages were made of Plexiglas material (65 x 25 x 15 cm) with the floor covered with sawdust. They were kept under standard dark-light cycle (lights on between 7:00 and 19:00 h), at a room temperature of 22±2°C. The rats had free access to food (standard rat chow) and water (or caffeine solution, see below), except for the stressed group, during the periods when restraint stress was applied. Both, stress and caffeine treatment, were kept until the end of the experiments. All animal treatments were in accordance to the institutional guidelines and to the recommendations of the International Council for Laboratory Animal Science (ICLAS), and of the Federation of Brazilian Society for Experimental Biology. All efforts were done to minimize animal suffering as well as to reduce the number of animals.

Caffeine Administration

Caffeine (Vetec, Rio de Janeiro, Brazil) was administered in drinking water as the only source of water during all the period of the experiment (Gasior et al., 2000). During the behavioral testing, the animals continued receiving caffeine treatment.

Control animals received tap water. The volume of water and caffeine solution consumed was measured every 48 hours. Studies from the literature using similar doses of caffeine in the drinking water found caffeine levels in the blood which were directly dependent of the dose consumed, since after chronic caffeine administration in concentrations of 0.25 g/L and 1.0 g/L resulted in plasma caffeine concentrations ranging from 1.45 to 5.95 µg/ml, respectively (Gasior et al., 2000).

Chronic restraint stress procedure

The animals were stressed 1 h/day, five days per week, for 50 days, when behavioral tests began. Restraint stress began at the same day caffeine treatment was initiated, and was carried out by placing the animal into a 25 x 7cm plastic tube, and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. The control group was not submitted to stress and animals were kept in their home cages. After this period, anxiety-like behavior was evaluated and restraint stress continued to be carried out. Restraint stress began at least 1h after behavioral procedures.

Elevated plus-maze test

The elevated plus maze test was conducted after 50 days of treatment, using a standard plus maze apparatus kept 80 cm above the floor, consisting of four arms arranged in the shape of a cross (arms measured 45 x 10 cm). The four arms were joined at the center by a 10 cm square platform. Two of the arms, opposite to each other, had no walls (open arms); the two other arms (closed arms) had 23-cm high walls. This test is considered sensitive to the anxiety state of the animal, based on the principle that

exposure to an elevated and open arm maze leads to an approach conflict that is stronger than that evoked by exposure to an enclosed arm maze (Pellow and File, 1986). On the day of the experiment, the animals were acclimatized to the behavioral testing room for 5 min prior to the initiation of the test. Animals were placed individually on the center of the maze, on the junction between open and closed arms, facing one of the open arms, and performance was scored during 5 min. A rat was considered to have entered one arm of the maze when all four feet were within the arm. Conventional parameters of anxiety-like behavior were monitored, i.e., the number of entries into the closed arms, entries into the open arms, total entries, and the total time spent in each arm. The ratio "time spent in the open arms/time spent in all (i.e., open and closed) arms" was calculated and multiplied by 100, to yield the percentages of time spent in open arms. This parameter is considered to reflect fear-induced inhibition from entering the open arms and can be related to the "anxiety" level experienced by the animals. Another anxiety index was obtained by dividing the number of entries into open arms by the number of entries into open plus closed arms and multiplying by 100.

Exposure to the Open Field

Ten days after exposure to the plus maze, the animals were exposed to an open field. A 50 cm-high, 40 x 60cm open field made of brown plywood with a frontal glass wall was used (Mello e Souza et al., 2000). The floor was subdivided with white lines into 12 equal 13.3 by 15.0 cm rectangles. Measurements were taken in a brightly lit room, set up so that uniform light was applied on the floor of the open field. The animals were gently placed facing the left corner and allowed to explore the arena for 3 min. The line crossings (ambulation) was counted. The open field was washed with 5%

ethanol before a new animal was introduced. Twenty-four hours later, the animals were again exposed to the same apparatus. Crossings and time spent in the central part of the open field were evaluated.

Blood collection and adrenal dissection

Caffeine and chow were maintained until the moment of the sacrifice. Eighteen days after the behavioral procedures, animals were sacrificed by decapitation between 12:00 and 14:00 hours, at least 24 hours after the last restraint exposure, and the trunk blood was collected into heparinized tubes, centrifuged at 4^{o}C at 1,000 g, and plasma separated and stored at -70^{o} C for corticosterone determination. All animals were killed within this interval of time in a random order considering stressed and non-stressed animals. Adrenal glands were carefully dissected and weighed using a scale with a precision of 0.0001g.

Single cell gel electrophoresis — comet assay

A standard protocol for comet assay preparation and analysis was adopted (Tice et al., 2000). After the sacrifice of the animals by decapitation, the hippocampus was immediately dissected out and gently homogenized in phosphate-buffered saline solution (PBS) pH 7.4. The slides were prepared by mixing 20 µl of hippocampus homogenate (in cold PBS), with 80 µl low melting point agarose (0.75%). The mixture (cells-agarose) was added to a microscope slide coated with a layer of 500 µl of normal melting agarose (1%). After solidification, the cover slip was gently removed and the slides were placed in lysis solution (2.5M NaCl, 100 mM EDTA and 10 mM Tris, pH

10.5, with freshly added 1% Triton X-100 and 10% DMSO) for one day. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 10 min. The DNA was electrophoresed during 20min at 25V (0.90 V/cm) and 300mA. Afterwards, the slides were neutralized with Tris buffer (0.4 M; pH 7.5). Finally, the DNA was stained with ethidium bromide. After electrophoresis, neutralized and stained nuclei (from random 100-cells fields) were blindly analyzed by fluorescence microscopy with visual inspection (200x). Cells were scored from zero (undamaged) to 4 (maximal damage), according to the tail intensity (size and shape), resulting in a single DNA damage score for each cell, and, consequently, for each group. Therefore, a group damage index could range from zero (all cells no tail, 100 cellsx0) to 400 (all cells with maximally long tails, 100 cellsx4) (Collins et al., 1997) (Figure 1). The DNA damage index was calculated by multiplying the number of cells by its respective index score and than summing up.

- Insert Figure 1 about here –

Corticosterone determination

For corticosterone determination, plasma was extracted with ethyl acetate, the extract evaporated and the residue suspended for the hormone evaluation with an ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA).

Statistical analysis

Data were expressed as means ± standard error of the mean (S.E.M), and were analyzed using two-way ANOVA (factors were stress and caffeine) followed by the

Duncan multiple range test, when indicated. Significance level was accepted as different when the P value was equal or less than 0.05.

Results

Caffeine consumption

There were no statistical differences between control and stressed groups on drinking volumes consumed, therefore mean caffeine consumption did not differ between stressed and control animals, neither in males nor in females. Rats of both sexes receiving caffeine 0.3g/L consumed about 40 mg/Kg/24 h and rats receiving 1.0 g/L consumed about 108 mg/Kg/24 h. Fluid consumption was around 37 ml/day for males and 27.5 ml/day for females.

Adrenal weight and corticosterone measurements

A two-way ANOVA revealed no differences between the groups regarding adrenal weight (data not shown; $P>0.05$). However, on corticosterone measurements, female animals receiving caffeine 1.0g/L showed a decrease in plasma corticosterone levels [caffeine effect, $F(2,24) = 3.75$, $P<0.05$]. No differences were observed in male animals on this measurement (Figure 2).

- Insert Figure 2 about here -

Effect of chronic consumption of caffeine and chronic stress on behavior in the open field and in the elevated plus-maze test

No significant difference was observed between the groups in the number of crossings during this task, neither in male nor in female animals (Figure 3). In male animals caffeine induced a reduction on time spent in the central part of the open field [$F(2,58) = 5,466$, $P<0.01$], while no effect of stress [$F(1,58) = 0.044$, $p>0.05$] or interaction [$F(2,58) = 0.323$, $p>0.05$] were observed. On the other hand, no differences in the time spent in the central area were observed in females (Figure 4).

The results concerning the behavior of the rats in the elevated plus-maze are shown in Table 1. For male rats, both chronic stress [$F(1,58) = 7,406$, $P<0.01$] and caffeine treatment [$F(2,58) = 3.141$, $p=0.05$] caused a decrease in the number of entries in open arms. No difference in the number of entries in closed arms, as well as in the number of total entries, was found. Both chronic stress [$F(1,58) = 4.099$, $P<0.05$] and caffeine treatment [$F(2,58) = 4.813$, $P<0.05$] also induced a decrease in the percentage of time spent in open arms. Accordingly, there was an increase in the time spent in the closed arms [$F(1,58) = 4.086$, $P<0.05$ for the stress effect and $F(2,58) = 4.07$, $P<0.05$ for the caffeine effect]. No interactions were observed between stress and caffeine treatment in these parameters ($P > 0.05$). No significant difference was observed in female performance in any of these parameters (Table 1).

- Insert Figures 3 and 4 about here -

- Insert Table 1 about here -

DNA damage in the hippocampus

Results concerning DNA damage index in the hippocampus are displayed in Figure 5. Both chronic stress [$F(1,27) = 20.818$, $P<0.001$] and caffeine treatment [$F(2,27) = 12.066$, $P<0.001$] induced an increase on DNA damage in male rats. Additionally, there was an interaction between caffeine and stress [$F(2,27) = 3.878$, $P<0.05$], since the effect of caffeine on this parameter does not sum up with the stress effect. On the other hand, a pilot study showed no differences between the groups on DNA damage in the hippocampus of female rats (data not shown).

- Insert Figure 5 about here -

Discussion

The major findings in this work are that chronic stress and caffeine treatment effects on anxiety-like behavior and on DNA damage in the hippocampus are sex-specific. Both treatments increased anxiety-like behavior, and also lead to increased DNA damage in the hippocampus of male rats; however, females were more resistant to those treatments. Furthermore, an interaction between, stress and caffeine intake, was also observed.

Corticosterone levels were only modified in female animals consuming caffeine 1.0g/L, when a decrease in plasma corticosterone levels was found. It has been observed that high doses of caffeine produce a stress-like neuroendocrine response in rats, which is characterized by increased serum corticosterone (Spindel, 1984); however, tolerance

to the effects of caffeine on corticosterone levels was developed in seven days (Spindel et al., 1983). In the present study a chronic treatment of more than 40 days was used, therefore it is possible that the reduced corticosterone levels observed here are result of the chronicity of the treatment. Regarding the behavioral analysis, no effect was observed on motor activity in the open field task, showing development of tolerance against motor effects induced by caffeine intake (Antoniou et al., 2005; Karcz-Kubicha et al., 2003). On the other hand, in the plus maze task, male rats from groups receiving caffeine (1.0g/L and 0.3g/L) and from stressed groups presented an increase of anxiety-like behavior. The increased anxiety induced by chronic caffeine could also be observed when evaluating time spent in the central area of the open field, another parameter of anxiety-like behavior. In this task, however, stressed animals presented no difference.

The effect of chronic stress on anxiety, as observed in the plus maze task, agrees with other reports, showing that restraint stress may induce anxiety-like behavior in the plus maze task, and that these changes are more marked in males than in females (Chakraborti et al., 2007). In that study, neurochemical alterations (lipoperoxidation) were also more evident in males than in females.

The involvement of the adenosinergic system in anxiety related behaviors has been observed, and it has been suggested that adenosine, acting on A1 receptor, shows anxiolytic effect, but acting on A2 receptor promotes opposite effects (Kulkarni et al., 2007). Since caffeine blocks both sub-types of adenosine receptor (A1/A2), but induces anxiogenic effects when chronically administered, we may suggest that this effect could be due to its antagonism on A1 receptor (Correa and Font, 2008); however, adaptive alterations in the receptor levels should not be discarded (Johansson et al., 1997). Other reports from the literature also agree with our results concerning caffeine and anxiety: caffeine acutely or chronically administered induced anxiety-like effects in the plus

maze test. On the other hand, it was demonstrated that the acutely administration of non-xanthine A2A adenosine receptor antagonists ZM241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol) and SCH58261 (5-amino-7-(β-phenylethyl)-2-(8-furyl)pyrazolo[4,3-e]-1,2,4-triazolo-[1,5c]pyrimidine) are devoid of activity in the plus-maze test in mice at behaviorally active doses. In this report it was suggested that acute effects of caffeine may not be solely due to an impact upon the A2A receptor (El Yacoubi et al., 2000). Interestingly, caffeine has also been shown to present antidepressant activity. Selective adenosine A2A receptor antagonists were effective in reversing signs of behavioral despair in the tail suspension and forced swim tests, procedures predictive of antidepressant activity (El Yacoubi et al., 2003). Although the mechanism relating caffeine intake and anxiety is not completely understood, it is important to point that the anxiogenic effect of caffeine treatment was observed in this study even after a chronic treatment, at least in males.

Interestingly, in this study sex-specific differences were found, since female rats appear to be more resistant than males to the effects of caffeine and stress on the evaluated parameters. This observation agrees with other findings, when female rats were observed to be insensitive to chronic stress in terms of facilitation and consolidation of anxiety (Mitra et al., 2005). It has been suggested that gonadal hormones, most likely estradiol (E2), may contribute to the resistance of adult female rats to chronic stress (Luine et al., 2007), since it may act as anxiolytic agents (Hill et al., 2007). Studies shown that ovariectomy, by removing the primary source of E2, the ovaries, increases anxiety and depressive behavior, and acute, subchronic, or chronic physiological E2-replacement reduces anxiety and depressive behavior of rodents (Bernardi et al., 1989; Bowman et al., 2002), although E2's effects on anxiety of rodents may depend upon the E2 dosage and/or regimen (Walf and Frye, 2005). Different

phases of the estrous cycle can influence female behavior. This cycle was not monitored in the present study, and probably females were tested in different estrous cycle phases, what would explain the higher standard deviation observed in those groups.

On male rats, caffeine consumption and chronic stress increased DNA damage in hippocampus. Caffeine is an alkaloid and its catabolic products, theobromine and xanthine, can reduce copper, from Cu(II) to Cu(I), leading to the generation of oxygen radicals "in vitro" (Shamsi and Hadi, 1995). The generation of oxygen radicals, increasing oxidative stress, may result in DNA breaks (Azam et al., 2003). Additionally, caffeine is an efficient inhibitor of DNA double-strand repair "in vitro" (Selby and Sancar, 1990), which may help to explain the increased DNA damage in hippocampus after caffeine consumption. However, strand breaks in DNA arise from oxidative damage, but also from the process of DNA repair (Halliwell and Whiteman, 2004), and this increased index of DNA breaks, as observed here, does not mean these breaks are permanent. Further studies to evaluate permanent damage are warranted, for a better understanding of chronic caffeine effect.

About stress and DNA damage it has been shown that exposure to repeated stress induces oxidative stress, especially in hippocampus (Fontella et al., 2005). This oxidative stress indicates production of ROS, such as peroxides, hydroxyl and superoxide anion radicals, which may induce cellular oxidative damage through DNA strand breaks and lipid peroxidation (Muqbil et al., 2006). Using restraint stress, Muqbil et al. (2006) observed no significant damage to DNA of lymphocytes, liver and skin cells of rats, however the stressor that they used seemed to prime the cells to respond to subsequent induction of DNA damage, either by impairing the ability of cells to repair DNA, or by causing oxidative stress. In addition, it is possible that the hippocampus may be more sensitive to DNA damage since many endangering effects of stress are

most pronounced in this structure, probably due to its high concentrations of corticosteroid receptors and sensitivity to glucocorticoids (Sapolsky, 1996). Additionally, the longer period of stress exposure in the present study, when compared to Muqbil et al. (2006), may have contributed to generate the increase in DNA damage.

An interaction between stress and caffeine on DNA damage was detected. When these two factors were combined, the effect was not higher as it would be expected from their separate effects. This could be due to a ceiling effect, or to some inhibitory role of caffeine on the stress response; this last possibility, however, is not supported by plasma corticosterone measurements since the inhibitory effect of caffeine on the stress response axis was observed only in females, at least when considering corticosterone levels.

In conclusion, this study verified an increase on anxiety-like behavior and on DNA damage in male rats, induced by chronic caffeine and stress. Female rats were more resistant to these effects; these results may be related to the presence of estradiol, which may have anxiolytic and neuroprotective properties. To our knowledge, this is the first study showing the effects of chronic stress and chronic consumption of caffeine (together) on DNA damage in the brain of the rats. These results contribute to understand the sex-specific differences on these parameters, as well as to suggest that further studies are necessary to elucidate the mechanisms involved in these processes.

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Table 1

Effects of chronic consumption of caffeine and chronic stress on behavioral parameters measured in the elevated plus-maze test.

Group	Non-stressed			Stressed		
	Control	Caffeine 0.3g/L	Caffeine 1g/L	Control	Caffeine 0.3g/L	Caffeine 1g/L
Males						
Time in open arms[a]	145.8±16.5	96.1±15.5	97.6±19.9*	111.1±15.8	88.5±20.1	52.0±15.5*
Time in closed arms[a]	104.9±13.8	142.6±10.3	143.9±18.2*	134.2±15.6	147.9±14.1	185.6±21.6*
% time in open arms[a]	57.5±5.9	38.7±5.4*	39.6±7.3*	45.2±6.1	34.7±7.4*	22.4±6.8*
Entries in open arms[a]	8.0±0.8	7.8±1.3	5.5±0.7*	6.4±1.0	4.6±0.7	3.6±0.9*
Entries in closed arms	7.6±0.7	8.1±0.8	8.3±1.7	7.7±0.9	7.5±0.8	9.3±1.6
Total entries	15.6±0.9	15.9±1.6	13.8±1.4	14.1±1.6	12.2±1.1	12.9±2.1
% open entries[a]	51.0±3.8	46.5±4.6	42.9±6.3	42.8±4.9	37.6±4.3	28.4±6.0
Females						
Time in open arms	150.9±21.7	121.3±18.4	119.1±28.9	115.4±18.1	122.8±21.7	106.8±17.4
Time in closed arms	108.9±15.8	140.9±13.0	139.0±26.9	150.1±18.6	135.6±19.8	153.5±12.5
% time in open arms	56.9±7.3	45.3±6.6	45.3±10.2	43.5±6.9	47.1±7.5	40.2±6.0
Entries in open arms	10.9±1.4	8.1±1.3	7.8±1.9	8.9±1.2	9.0±1.7	7.9±1.3
Entries in closed arms	7.5±0.7	6.8±0.7	6.1±1.3	8.0±1.1	8.0±1.4	8.6±1.2
Total entries	18.4±1.3	14.9±2.0	13.9±2.5	16.9±1.5	17.0±2.5	16.5±0.7
% open entries	57.6±5.4	51.7±5.1	49.0±9.8	52.3±5.0	52.5±5.0	47.6±7.4

Data are expressed as mean ± S.E.M. for each parameter.

Male, N=7-13/group. Female, N= 8/group.

[a]Significant difference between stressed and control animals (two-way ANOVA, $P \leq 0.05$). *Different from control (Duncan, $P < 0.05$)

Legends of the figures

Figure 1

Some typical examples of Comet assay. Evaluation of DNA damage using ethidium bromide (200×). The cells are assessed visually and received scores from 0 (undamaged) to 4 (maximally damaged), according to the size and shape of the tail. Scores were obtained using the mean score of two independent blind evaluators.

Figure 2

Corticosterone measurement in stressed and non-stressed animals. Data is expressed as mean $\pm$ S.E.M. A. Males, N=6-7/group. Two-way ANOVA shown no effect. B. Females, N = 4-6/group. Two-way ANOVA shown an effect of caffeine group ($p<0.05$)

* Significantly different compared to caffeine 0.3g/L and control, stressed or not. (Duncan multiple range test, $p < 0.05$).

Figure 3

Number of crossings in the open field in stressed and non-stressed animals. Data is expressed as mean $\pm$ S.E.M. A. Males, N = 7 -13/group. Two-way ANOVA showed no effect. B. Females, N = 8/group. Two-way ANOVA showed no effect.

Figure 4

Time spent in the central area of the open field in stressed and non-stressed animals. Data is expressed as mean ± S.E.M. A. Males, N = 7 -13/group. Two-way ANOVA showed an effect of caffeine in this parameter ($P < 0.01$).

*Animals treated with both doses of caffeine were different from animals not receiving caffeine (Duncan multiple range test, $p < 0.05$). B. Females, N = 8/group. Two-way ANOVA showed no effect.

Figure 5

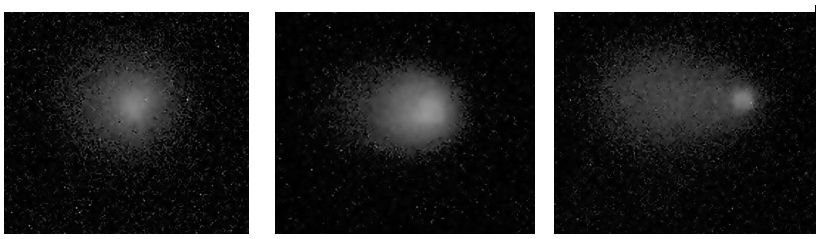
DNA damage in hippocampus by Comet Assay in stressed and non-stressed male animals. Data is expressed as mean ± S.E.M. Males, N = 3-7/group. Two-way ANOVA showed effect of caffeine ($p<0.001$) and stress ($p<0.001$) groups.

There was a significant interaction between stress and caffeine treatment ($P < 0.05$).

* Significantly different compared to non-stressed.

Significantly different compared to control, stressed or not (Duncan multiple range test, $p < 0.05$).

Figure 1



No damage Damage 2 Damage 4

Figure 2

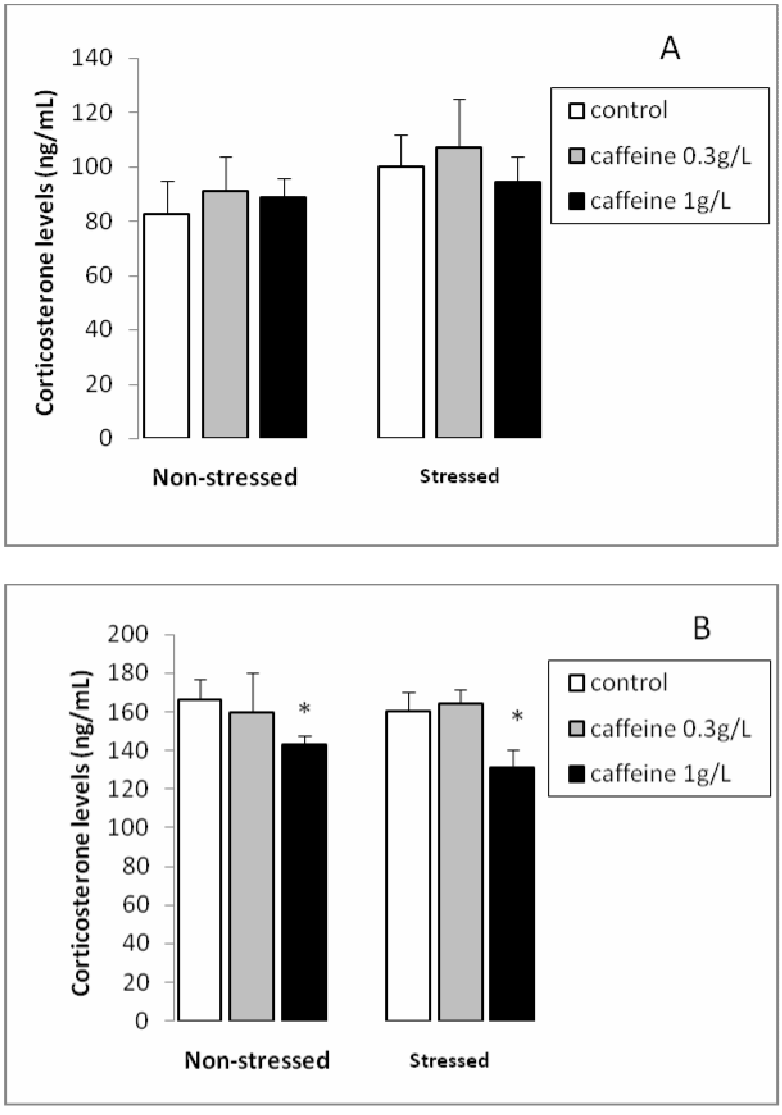
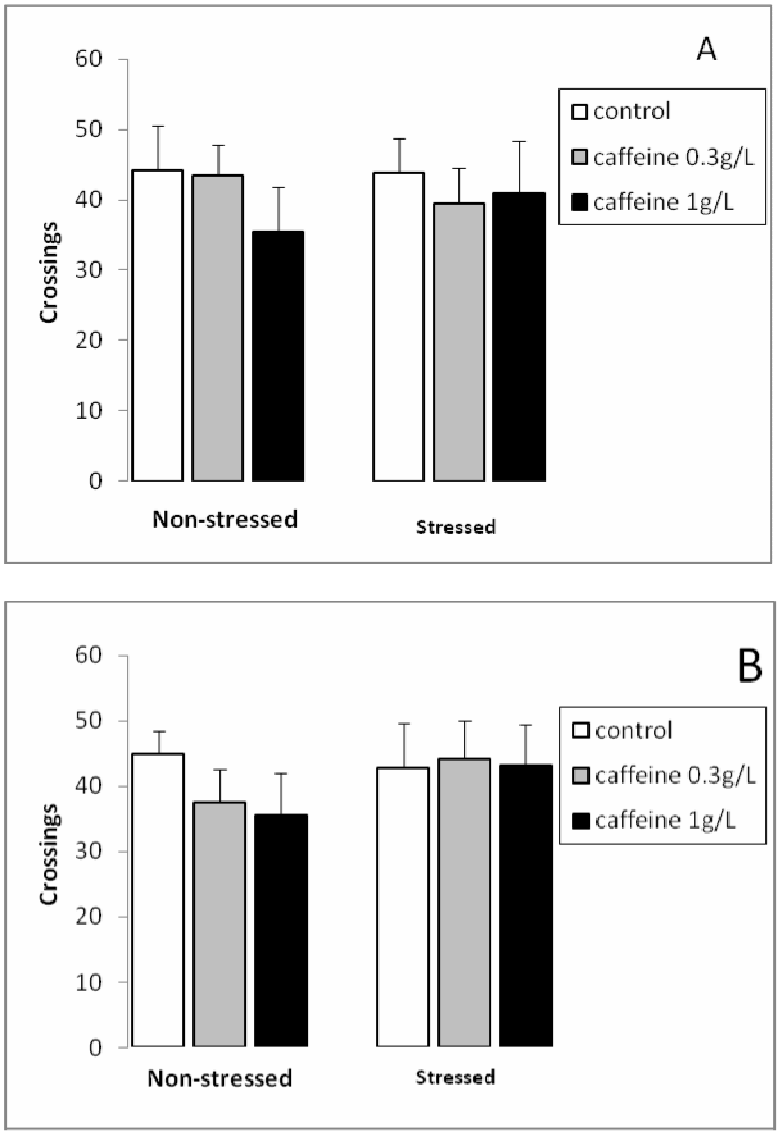


Figure 3


A
60
50
40
30
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Crossings
Non-stressed
Stressed
control
caffeine 0.3g/L
caffeine 1g/L
B
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Crossings
Non-stressed
Stressed
control
caffeine 0.3g/L
caffeine 1g/L

Figure 4

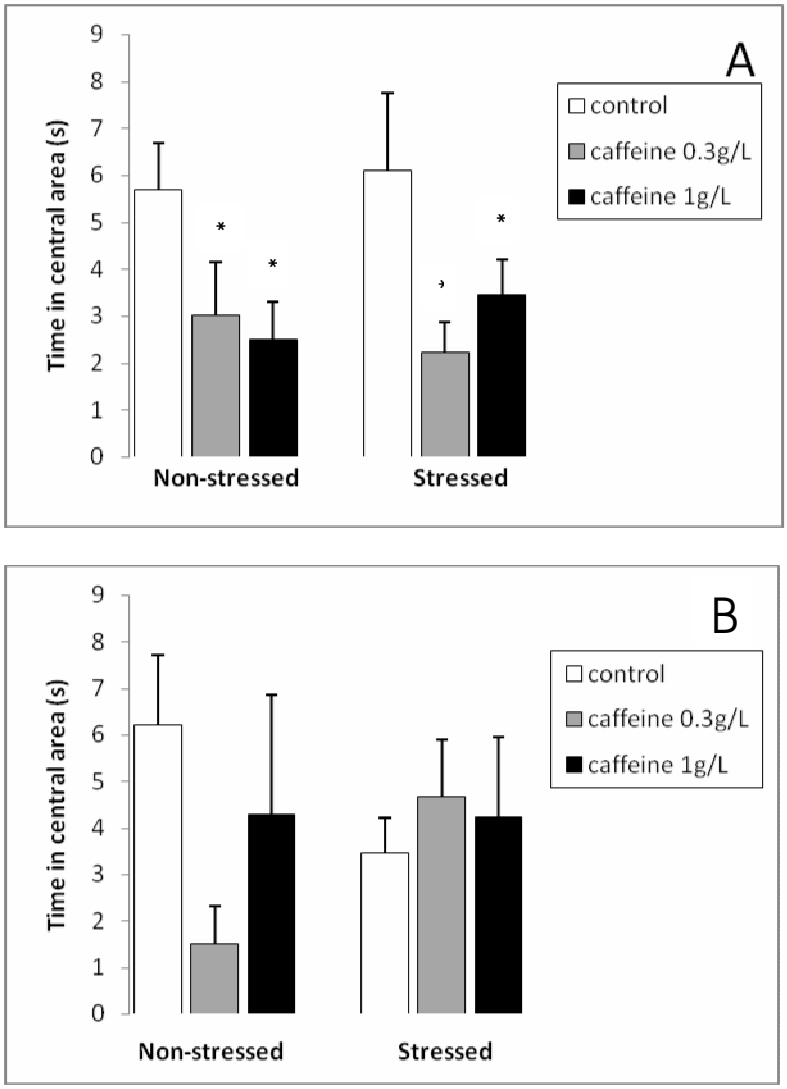
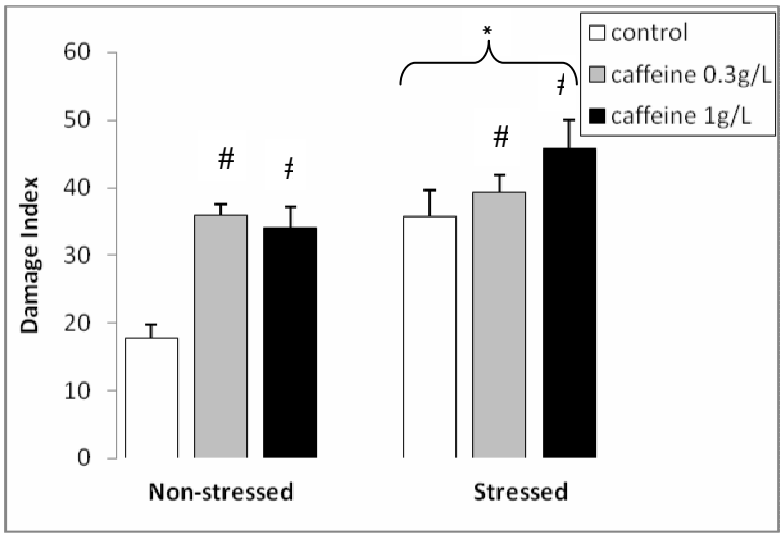


Figure 5



3.2 Capítulo 2

Interactions between chronic stress and chronic consumption of caffeine on the enzymatic antioxidant system

Artigo submetido para publicação na revista Neurochemical Research.

INTERACTIONS BETWEEN CHRONIC STRESS AND CHRONIC CONSUMPTION OF CAFFEINE ON THE ENZYMATIC ANTIOXIDANT SYSTEM

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Suggested running head: Chronic stress and caffeine consumption: interactions on antioxidant system

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Abstract

We studied the effect of chronic caffeine on parameters related to oxidative stress in different brain regions of stressed and non-stressed rats. Wistar rats were divided into 3 groups: control (receiving water), caffeine 0.3 g/L and caffeine 1.0 g/L (in the drinking water). These groups were subdivided into non-stressed and stressed (repeated restraint stress during 40 days). Lipid peroxide levels and the total radical-trapping potential were assessed, as well as antioxidant enzyme activities [superoxide dismutase (SOD), gluthatione peroxidase (GPx) and catalase (CAT)] in hippocampus, striatum and cerebral cortex. Results showed interactions between stress and caffeine, especially in the cerebral cortex, since caffeine increased the activity of some antioxidant enzymes (SOD and CAT), but not in stressed animals. We concluded that chronic administration of caffeine led, in some cases, to increased activity of antioxidant enzymes. However, these effects were not observed in the stressed animals.

Keywords: oxidative stress, caffeine, chronic stress, antioxidant enzymes, brain structures.

Introduction

Caffeine is probably the world's most popular drug. It is taken regularly by most of the population through coffee, tea, cola drinks, cocoa and chocolate (1). Over the last years, accumulating evidences have suggested a potential neuroprotective role for caffeine, both in animal models of neurodegenerative diseases (2,3) and in epidemiological studies (4). For example, caffeine protects against neurotoxicity and degeneration of dopaminergic nigrostriatal system animal models of Parkinson Disease (2,3), and, in humans, an inverse relationship between caffeine consumption and the risk of developing Parkinson or Alzheimer Diseases has been observed (4,5). In addition, caffeine may have protective effects inhibiting carcinogenesis induced by chemical agents (6). The biochemical mechanism that underlies the actions of caffeine at relevant doses to the daily intake of coffee (at μM range) is mediated by adenosine receptors blockade, although at high doses (at mM range) it may inhibit phosphodiesterase, and mobilize intracellular calcium (7).

Caffeine and its metabolites have been shown to present antioxidant effects (8) (9,10), and these properties may be related to its neuroprotective actions. Most of these studies relating caffeine and oxidative stress were performed in vitro, but a few studies using chronic caffeine administration have also suggested its potential role in increasing antioxidant enzymes activities in liver and heart (11,12). Since chronic caffeine consumption has been suggested to decrease the risk for some neurodegenerative diseases, it would be interesting to consider the effect of chronic use of caffeine on parameters related to oxidative stress in brain regions.

Reactive oxygen species (ROS) are believed to be involved in tissue damage resulting from a wide variety of insults. These substances can directly damage cellular

proteins, DNA, and lipids, and thereby affect all cellular functions (13). In order to neutralize the effects of reactive species, the cell uses antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (14). The brain is especially vulnerable to free radical-induced damage because of its high oxygen consumption, abundant lipid content and relative paucity of antioxidant enzymes (14) (15), and oxidative injury has been proposed as an etiopathology of several CNS disorders (16).

It is well known that chronic exposure to stress situations leads to a series of biochemical, physiological and behavioral changes, thus, altering normal body homeostasis (17). Altered activities of the antioxidant defense system enzymes and levels of free radical scavengers, as well as other parameters of oxidative stress, have been found to be correlated with conditions related to stress exposure (18-21), suggesting that the stress response leads to increased production of free radicals (22) (21,23).

Stress is part of modern society life and many times it is associated with caffeine consumption. As caffeine may have antioxidant properties (9) and stress seems to cause oxidative damage (18), the aim of this study is to verify the effect of the chronic consumption of caffeine on the antioxidant enzymes activities, total reactive antioxidant potential and on lipid peroxidation in brain structures of male rats, comparing the effects of caffeine in stressed and non-stressed rats.

Experimental Procedure

Animals

For the present study, 56 adult male Wistar rats from our breeding stock (60 days of age at the beginning of the treatment), weighing 250-300g were used. The animals were divided into three groups: control (receiving water), caffeine 0.3 g/L and caffeine 1.0 g/L (the last two groups receiving caffeine in drinking water). These groups were subdivided into non-stressed and stressed (repeated restraint stress during 40 days). The final groups were: control (non-stressed receiving water), caffeine 0.3g/L (non-stressed receiving caffeine 0.3g/L), caffeine 1.0g/L (non-stressed receiving caffeine 1.0g/L), stressed (stressed receiving water), stressed + caffeine 0.3g/L (stressed receiving caffeine 0.3g/L) and stressed + caffeine 1.0g/L (stressed receiving caffeine 1.0g/L), resulting in 6 groups. The animals were housed in groups of three to five, in home-cages made of Plexiglas material (65 x 25 x 15 cm) with the floor covered with sawdust. They were kept under standard dark-light cycle (lights on between 7:00 and 19:00 h), at a room temperature of 22±2°C. The rats had free access to food (standard rat chow) and water (or caffeine solution, see below), except for the stressed group, during the periods when restraint stress was applied. All animal treatments were in accordance to the institutional guidelines and to the recommendations of the International Council for Laboratory Animal Science (ICLAS), and of the Federation of Brazilian Society for Experimental Biology. All efforts were done to minimize animal suffering as well as to reduce the number of animals.

Caffeine Administration

Caffeine (Vetec, Rio de Janeiro, Brazil) was administered in drinking water as the only source of water during all the period of the experiment (24). Control animals received

tap water. The volume of water and caffeine solution consumed was measured every 48 hours. Studies from the literature using similar doses of caffeine in the drinking water found caffeine levels in the blood which were directly dependent of the dose consumed, since after chronic caffeine administration in concentrations of 0.25 g/L and 1.0 g/L resulted in plasma caffeine concentrations ranging from 1.45 to 5.95 µg/ml, respectively (24).

Chronic restraint stress procedure

The animals were stressed 1 h/day, five days per week, for 40 days. Restraint was carried out by placing the animal into a 25 x 7cm plastic tube, and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. The control group was not submitted to stress and animals were kept in their home cages.

Preparation of the Samples

The animals were killed by decapitation 24 h after the last exposure to stress. Their hippocampi, striatum and cortex were quickly dissected out and were stored at minus 70ºC until analysis, when they were homogenized in 10 vol (w:v) ice-cold 50mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA for determination of Total Reactive Antioxidant Potential (TRAP), Superoxide Dismutase (SOD) Activity, Glutathione Peroxidase (GPx) Activity. For the determination of TBARS the samples were homogenized in 10vol (w:v) ice-cold 20mM potassium phosphate buffer (pH 7,4),

containing 140 mM KCl. On the other hand, to determinate CAT activity, samples were homogenized in 10vol (w:v) ice-cold potassium phosphate buffer 10mM (pH 7,0).

The total homogenate was used in the determination of TBARS. For other determinations, the homogenate was centrifuged at 3,000 rpm for 10min at 4ºC and the supernatant was used.

Determination of Thiobarbituric Acid Reactive Substances - TBARS

The formation of thiobarbituric acid reactive substances (TBARS) is used as an indicator of lipoperoxidation. Malondialdehyde (MDA), a product of lipoperoxidation, reacts with two molecules of thiobarbituric acid (TBA) at low pH and high temperature to form a pink-colored complex (25). Therefore, the formation of TBARS is expressed as MDA equivalents/mg of protein. Aliquots of samples were preincubated for 15min on a 37ºC water bath, and 10% TCA was added. Samples were shaken and centrifuged at 3,000 rpm for 10min at 4ºC. The supernatant was removed and 0.67% TBA was added to it. The mixture was heated (30 min) on a boiling water bath. Afterwards, n-butanol was added and the mixture was shaken and centrifuged. The organic phase was collected and assayed spectrophotometrically at 532nm. 1,1,3,3-Tetramethoxypropane, which is converted to MDA, was used as standard.

Total Reactive Antioxidant Potential (TRAP) Assay

This assay is based on luminol-enhanced chemiluminescence measurement induced by an azo initiator (26). The reaction mixture contained 10 mM 2-2'azobis(2-amidinopropane)dihydrochloride (ABAP), a source of peroxyl radicals, and 0.01 mM

luminol in glycine buffer (0.1 M, pH 8.6). The chemiluminescence generated was measured in a scintillation counter (Beckman) working out of coincidence mode. The addition of Trolox (antioxidant standard) or samples decreased chemiluminescence to basal levels for a period (induction time) proportional to the concentration of antioxidants. The TRAP values were calculated as equivalents of Trolox per mg of protein and expressed as percent of control.

Superoxide Dismutase Activity

SOD activity was determined using a RANSOD kit (Randox Labs., USA) which is based on the procedure described by Delmas-Beauvieux et al. (27). This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a formazan dye that is assayed spectrophotometrically at 492 nm at 37ºC. The inhibition in production of the chromogen is proportional to the activity of SOD present in the sample.

Catalase Activity

Catalase (CAT) is an enzyme able to degrade peroxides, including hydrogen peroxide (H_2O_2), and its activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25ºC (28). CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per milligram of protein, using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$.

Glutathione Peroxidase (GPx) Activity

GPx activity was determined according to Wendel (29), with modifications. The reaction was carried out at 37ºC in 200 uL of solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione and 0.4 U glutathione reductase. The activity of GPx was measured taking tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as pmol NADPH oxidized per minute per mg protein.

Protein Assay

The total protein concentrations were determined using the method described by Lowry et al. (30) with bovine serum albumin as the standard.

Statistical analysis

Data were expressed as means ± standard error of the mean (S.E.M), and were analyzed using two-way ANOVA (factors were stress and caffeine) followed by the Duncan multiple range test, when indicated. Significance level was accepted as different when the P value was equal or less than 0.05.

Results

There were no statistical differences between control and stressed groups on drinking volumes consumed, therefore, mean caffeine consumption did not differ between stressed and control animals. Rats receiving caffeine 0.3g/L consumed about 40 mg/Kg/24 h and those receiving 1.0 g/L consumed about 108 mg/Kg/24 h.

Two-way ANOVA presented no effect of caffeine or stress factors regarding total reactive antioxidant potential (TRAP) in the hippocampus ($P>0.05$). However, a tendency to an interaction was observed between stressed and caffeine treated animals [$F(2,30) = 3.07$; $P = 0.061$] (Table 1), since caffeine 0.3 appears to increase TRAP but only in control animals. No effect was observed in TBARS in this structure ($P > 0.05$, two-way ANOVA; see Table 2). On the antioxidants enzymes activities (SOD, CAT, GPx), no differences were found among the groups ($P>0.05$; Figure 1), however, exposure to chronic stress induced an increased ratio SOD:CAT in hippocampus [$F(1, 21) = 5.37$; $P < 0.05$].

In striatum, a two-way ANOVA showed no differences between the factors on TRAP and on TBARS determinations ($P>0.05$; see Tables 1 and 2). An effect of stress exposure was shown on the antioxidant enzymes activities, since stress increased SOD activity [$F(1, 28) = 6.66$; $P<0.05$] (Figure 2A), while showing a tendency to decrease CAT activity [$F(1, 23) = 3.40$; $P=0.078$] (Figure 2B). On the other hand no differences were found on GPx activity ($P>0.05$) (Figure 2C).

Two-way ANOVA showed no differences between the groups on TRAP and on TBARS determinations in cerebral cortex ($P>0.05$; Tables 1 and 2). On the antioxidant enzymes activities, two-way ANOVA showed an interaction between stress and caffeine on SOD activity [$F(2, 22) = 3.92$; $P<0.05$] (Figure 3A), and on CAT activity [$F(2, 23) = 5.76$; $P<0.01$] (Figure 3B), since the effect of caffeine increasing these

activities was observed only in animals not exposed to chronic stress. Two-way ANOVA also presented an effect of stress, decreasing GPx activity [$F(1,22) = 13.27$; $P<0.05$] (Figure 3C).

Discussion

The major findings in this work are that chronic stress and caffeine treatment cause changes on the antioxidant enzymes activities which differ depending on the brain structure, and when animals are exposed to both factors, stress and caffeine may act differently. A different vulnerability to oxidative stress in distinct regions of rat brain after exposure to stress has been suggested (23,31). Some studies have suggested a more pronounced vulnerability of the hippocampus (22), and other studies have observed that the cerebral cortex also present a high vulnerability (23,32).

Restraint stress has been shown to induce increased oxidative stress, and formation of ROS that are capable of damaging various body components (21,23,33). On the other hand, caffeine and its catabolic products theobromine and xanthine exhibit both antioxidant (8, 34) and pro-oxidant properties, “in vitro” (34). Here, no differences between the groups were found on TBARS determination. These results agree with some reports from the literature (31, 35), although several studies have shown increased TBARS in brain structures of chronically stressed animals (21,36,37). These differences may be related to the use of different models of chronic stress (different stressors and different periods of exposure to stress). Since the stress model used in the present study may be considered not severe stress and since it is repeated, adaptation may develop to this stressor. For example, after several exposures to restraint, we observed changes in

the behavior of the animals, including less defecation. Measurements of corticosterone have also been made previously and, although the chronically restrained rats still release corticosterone after a new restraint session, the increase in this hormone levels in plasma is much smaller than that presented in the first days of stress (38).

In the hippocampus, there was a tendency to an interaction between caffeine and stress on TRAP, because caffeine 0.3 g/L increased TRAP in control animals, while no effect was observed in stressed animals. This suggests that caffeine may have neuroprotective properties also increasing the antioxidant potential, but this effect depends on the dose, and also depends on the condition of the animals, since this protective effect was not observed in chronically stressed rats. Additionally, no effect was observed in the other structures analyzed.

No differences were found on the antioxidant enzymes activities in the hippocampus; however, exposure to chronic stress induced an increased ratio SOD:CAT. SOD is the enzyme responsible for converting superoxide radicals into hydrogen peroxide. The ROS scavenging activity of SOD is effective only when it is followed by the actions of CAT and GPX, because the dismutase activity of SOD generates hydrogen peroxide from the superoxide ion, which requires to be further scavenged by CAT and GPx (39) The hydrogen peroxide can interact with superoxide anion leading to the formation of the highly reactive hydroxyl radical (40), and this can also happen when hydrogen peroxide is in the presence of iron (39, 40). Therefore, it has been hypothesized that an imbalance in the SOD/CAT ratio might be responsible for oxidative alterations (41), and this result may suggest an increased vulnerability to oxidative stress in stressed animals hippocampus.

On striatum, stress increased SOD activity and there was a tendency to decrease CAT activity. This result suggests a possible exacerbation of ROS production in this brain structure, since these chemical species have been reported to directly increase SOD expression (42,43). In the same way as in hippocampus, the imbalance between these activities may favor the induction of oxidative stress when these cells are subjected to challenges. However, no damage was seen in lipids, maybe due to the action of GPx, which might have been enough to eliminate the hydrogen peroxide formed, even though there was no significant increase in GPx activity. Increased SOD activity in the striatum was also observed in other studies (44,45), as well as decreased CAT activity (45), although some of those authors did not found the same effect on CAT activity (44) maybe because in their work stress was applied in a different schedule.

In the cortex, there was an interaction between caffeine and stress on SOD and CAT activities, since caffeine was able to increase the activity of these enzymes in control animals, and presented no effect in stressed animals. Besides, there was a decrease in GPx activity in the stressed animals. This decrease was probably compensated by the increased CAT activity, neutralizing the hydrogen peroxide formed. In a more severe chronic stress model (6h/day during 21 days), a depletion of glutathione (GSH) was observed (46), further suggesting an impairment of the antioxidant system involving GSH after chronic stress.

In conclusion, we observed that repeated restraint stress induced a state of higher susceptibility to oxidative stress in some structures, considering the altered activities of antioxidant enzymes. On the other hand, the chronic administration of caffeine, that has been postulated as a neuroprotective substance (2,3), led, in some cases, to increased activity of antioxidant enzymes, suggesting a possible neuroprotective role. Since the

biochemical mechanism that underlies the actions of caffeine at these doses it is believed to be mediated by adenosine receptors blockade, it is possible that the altered antioxidant enzymes activities observed in this study may reflect caffeine influence on the function of these receptors. Nevertheless, it is important to consider that caffeine effects were not observed in the stressed animals. Therefore, the suggestion of caffeine administration to prevent some neurodegenerative diseases must consider the state of the subject, for stress may interfere in this effect. Studies concerning other effects of caffeine in stressed animals are warranted.

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Table 1

Effects of chronic consumption of caffeine and chronic stress on the Total Reactive Antioxidant Potential (TRAP) Assay.

Groups		Hippocampus	Striatum	Cortex
Non-stressed	Control	100.04 ± 3.28	100 ± 15.41	100 ± 14.28
	Caffeine 0.3g/L	118.74 ± 7.39	84.79 ± 4.39	98.99 ± 10.93
	Caffeine 1g/L	99.59 ± 6.79	95.24 ± 16.74	75.03 ± 10.29
Stressed	Control	108.87 ± 8.48	100.73 ± 19.39	81.75 ± 13.96
	Caffeine 0.3g/L	96.81 ± 5.29	104 ±16.15	78.98 ± 13.34
	Caffeine 1g/L	98.01 ± 5.36	83.71 ± 13.80	75.71 ± 15.78

Data are expressed as percent of control (mean ± S.E.M. for each parameter). N=5-9/group. A marginally significant interaction was observed between stressed and caffeine treatment (P = 0.061) in hippocampus.

Table 2

Effects of chronic consumption of caffeine and chronic stress on the determination of Thiobarbituric Acid Reactive Substances – TBARS

Groups		Hippocampus	Striatum	Cortex
Non-stressed	Control	0.97 ± 0.59	1.53 ± 0.22	1.71 ± 0.48
	Caffeine 0.3g/L	1.28 ± 0.22	1.20 ± 0.22	2.31 ± 0.31
	Caffeine 1g/L	1.28 ± 0.25	1.44 ± 0.48	1.80 ± 0.47
Stressed	Control	0.98 ± 0.32	1.29 ± 0.29	1.86 ± 0.47
	Caffeine 0.3g/L	1.46 ± 0.56	1.34 ± 0.28	2.32 ± 0.51
	Caffeine 1g/L	0.89 ± 0.18	1.05 ± 0.28	2.19 ± 0.36

Data are expressed as MDA equivalents (nmol/mg of protein); mean ± S.E.M. for each parameter. N=4-10/group.

Legends to figures

Figure 1

Effects of chronic restraint stress and chronic consumption of caffeine on antioxidant enzymes activities, (A) SOD, (B) CAT and (C) GPx in the hippocampus of rats. Data is expressed as mean $\pm$ S.E.M. N = 4-8/group. Two-way ANOVA showed no differences between the groups ($P>0.05$).

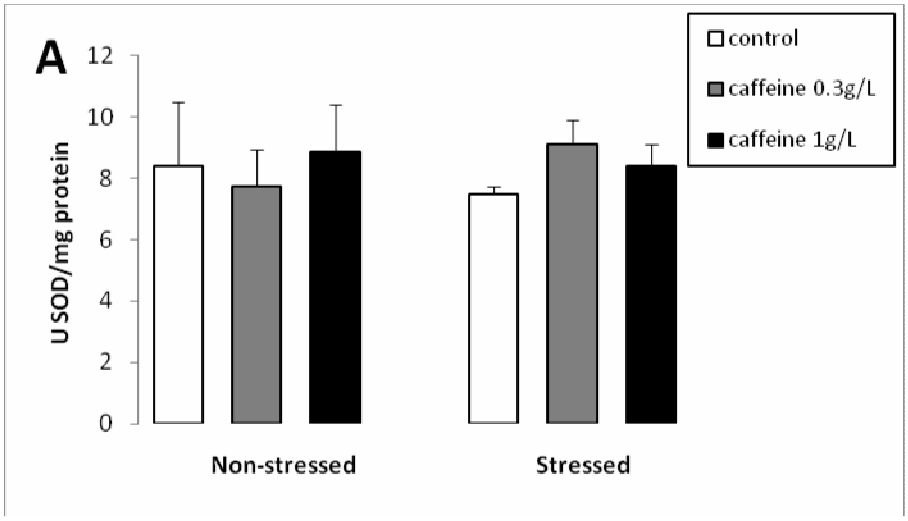
Figure 2

Effects of chronic restraint stress and chronic consumption of caffeine on antioxidant enzymes activities, (A) SOD, (B) CAT and (C) GPx in the striatum of rats. Data is expressed as mean $\pm$ S.E.M. N = 4-6/group. Two-way ANOVA showed an effect of stress, increasing SOD activity ($P<0.05$), and a marginally significant effect, decreasing CAT activity ($P =0.078$).

Figure 3

Effects of chronic restraint stress and chronic consumption of caffeine on antioxidant enzymes activities, (A) SOD, (B) CAT and (C) GPx in the cortex of rats. Data is expressed as mean $\pm$ S.E.M. N = 4-5/group. Two-way ANOVA showed an effect of stress on GPx activity ($P<0.05$). There were significant interactions between stress and caffeine treatment on SOD ($P<0.05$) and CAT activities ($P < 0.01$).

Figure 1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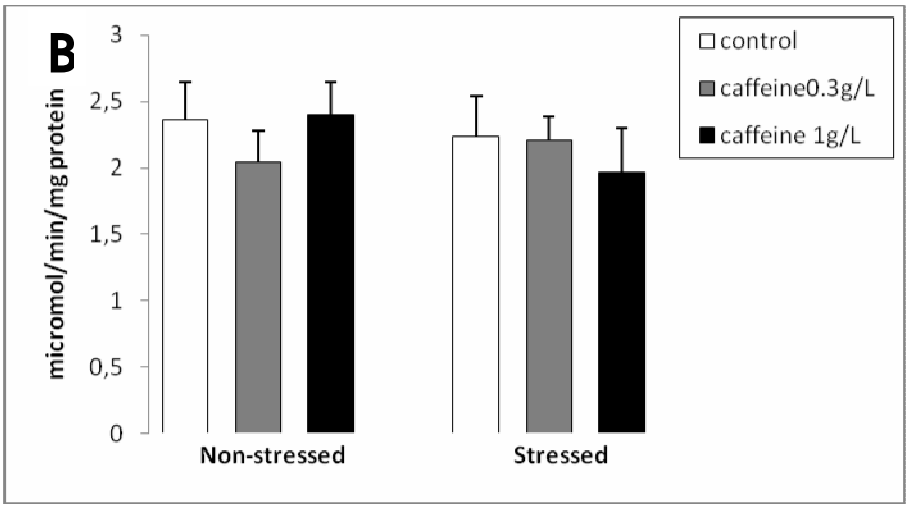
Non-stressed
Stressed
control
caffeine 0.3g/L
caffeine 1g/L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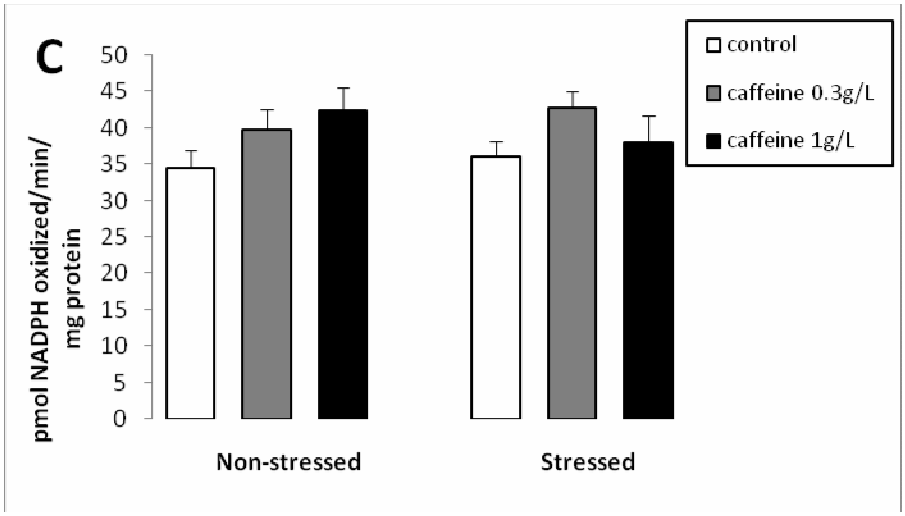
2,5
2
1,5
1
0,5
0
Non-stressed
Stressed
control
caffeine0.3g/L
caffeine 1g/L


C
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45
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Non-stressed
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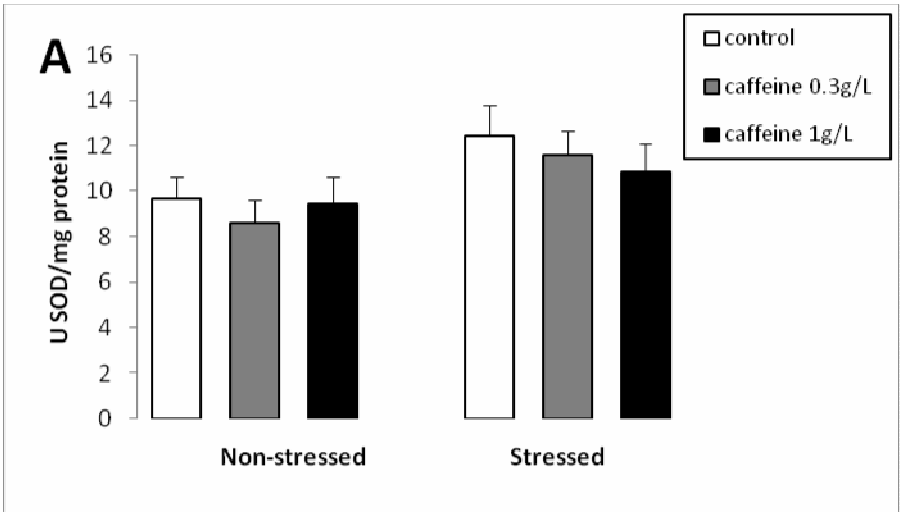
caffeine 1g/L

Figure 2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Non-stressed
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caffeine 0.3g/L
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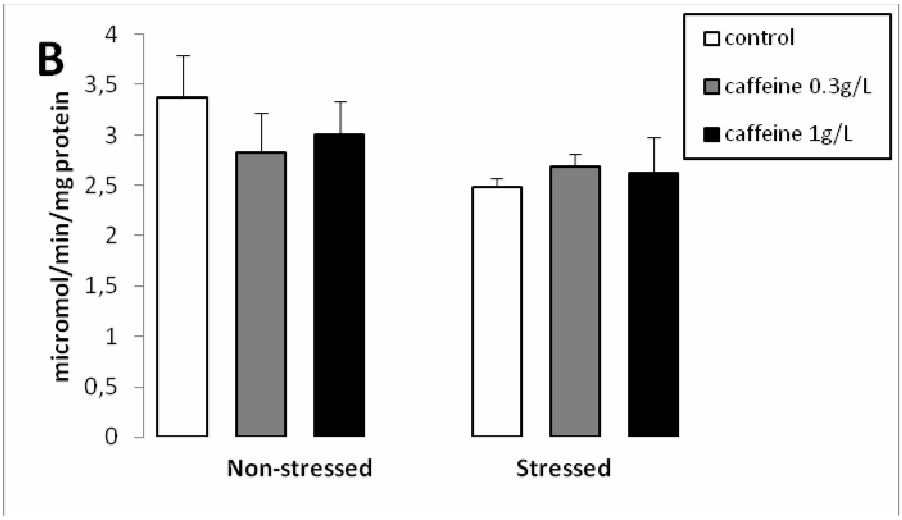
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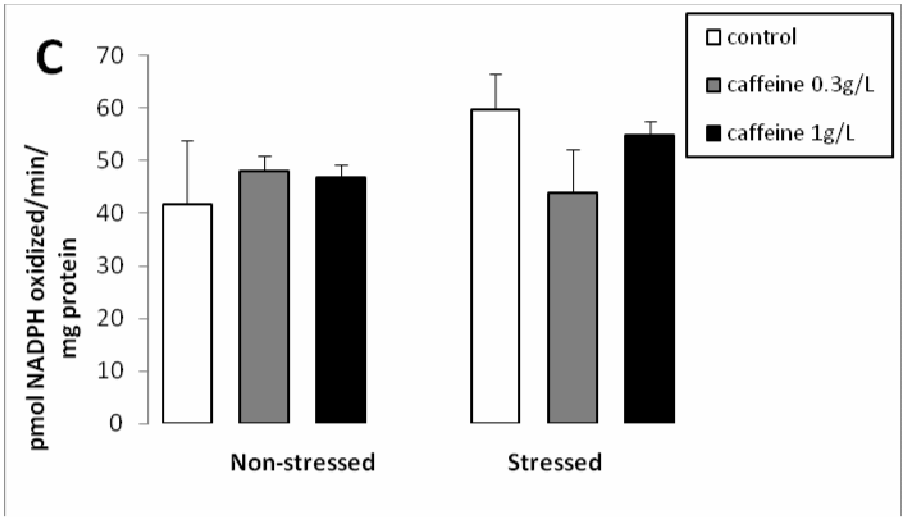
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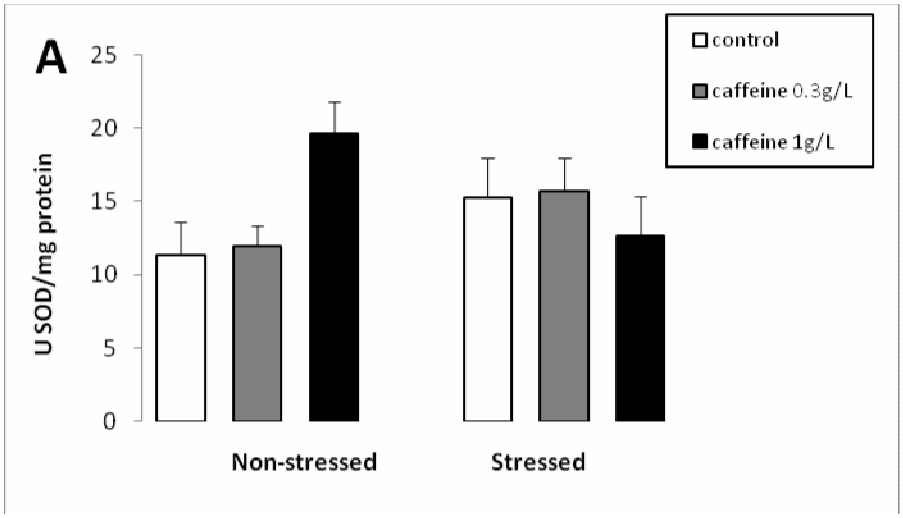
caffeine 0.3g/L
caffeine 1g/L

Figure 3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Non-stressed
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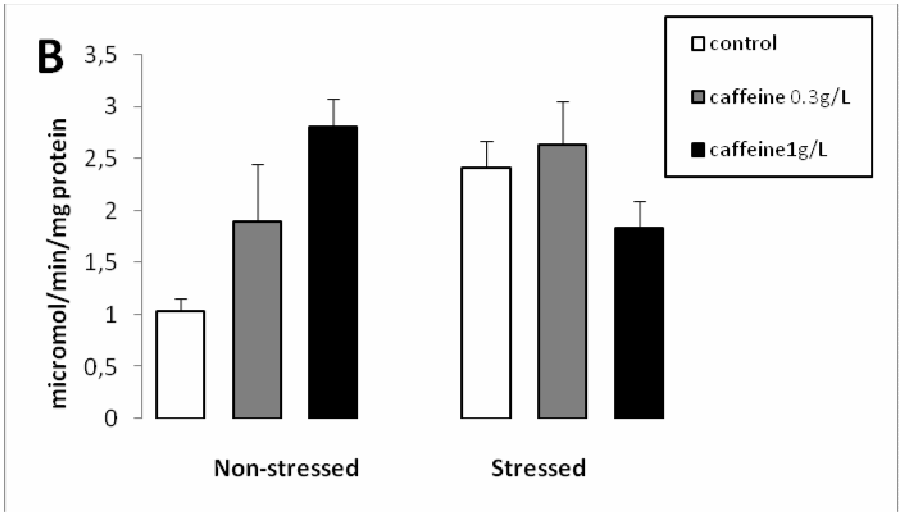
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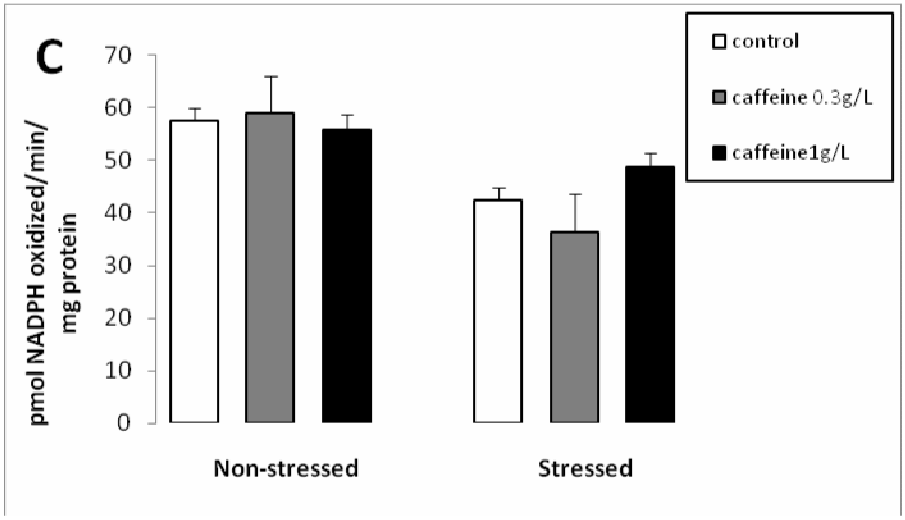
Non-stressed
Stressed


C
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control
caffeine 0.3g/L
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Non-stressed
Stressed

3.3 Capítulo 3

Efeito do consumo crônico de cafeína e do estresse crônico sobre o comportamento alimentar.

Tendo em vista que, o tipo, a duração ou a gravidade do estresse podem provocar diferentes respostas no comportamento alimentar (HARGREAVES, 1990; MARTI, MARTI e ARMARIO, 1994) e que a cafeína parece ter um pequeno efeito redutor da ingestão calórica (TREMBLAY ET AL., 1988), resolvemos estudar os efeitos do estresse crônico e do consumo crônico de cafeína sobre o comportamento alimentar em ratos.

3.3.1 Materiais e métodos

Animais

Formam utilizados 65 ratos Wistar adultos machos de 60 dias, pesando entre 250-300g. Os animais foram mantidos em caixas-moradia plásticas (65 X 25 X 15 cm) em grupos de 3 a 5, à temperatura de 22 $\pm$ 2°C e foram submetidos a um ciclo claro-escuro de 12 horas. Eles tinham livre acesso à comida (ração padrão) e à água ou à solução de cafeína, exceto para os animais estressados, durante o período em que a contenção foi aplicada. Os animais foram tratados de acordo com o recomendado pela SBNeC e com as recomendações do "International Council for Laboratory Animal Science (ICLAS)", buscando sempre minimizar o sofrimento bem como reduzir o número de animais utilizados.

Modelo de estresse e administração de cafeína

Foram feitos conforme descrito nos capítulos 1 e 2.

Exposição aos alimentos Froot Loops® e Cheetos®

No dia 41 de estresse, os ratos foram habituados a um novo ambiente contendo um novo alimento. Durante esse período eles foram colocados em uma caixa retangular de madeira (40cm X 15cm X 20cm) com vidro no teto. Dez Froot Loops (Kellogg's® - roscas de trigo, amido e sacarose) foram colocadas em uma extremidade da caixa. As sessões de treino (habituação) consistiram em 5 dias onde em cada dia os animais foram colocados individualmente na caixa por um período de 3 minutos, sob restrição alimentar (recebendo cerca de 80% da quantidade de ração normalmente ingerida) no dia anterior. Após a última sessão de habituação os animais foram alimentados *ad libitum* e foram submetidos à sessão de teste de 3 min, 24 horas depois. O tempo para começar a ingerir o alimento e a quantidade de alimento ingerida foram avaliados durante o treino (habituação) e no teste. Um protocolo foi estabelecido para quando o animal ingerisse parte do alimento (1/3 ou 1/4) essa fração fosse considerada. Para avaliar a exposição ao Cheetos (Elma Cheeps® – *pellet* a base de milho, queijo e sal) foram colocados 5 Cheetos® no mesmo aparato. As sessões de treinos (habituações) foram feitas por 3 dias, devido ao fato de o animal já estar familiarizado com o ambiente, e no 4º dia foi realizado o teste, da mesma maneira que foi descrito para o Froot Loops®.

Análise estatística

Os dados estão expressos como média $\pm$ E.P.M. e foram analisados por ANOVA de 2 vias no caso do ganho de peso e nos testes com os alimentos Froot Loops® e

Cheetos®. ANOVA de medidas repetidas foi utilizada na análise dos treinos com estes alimentos.

3.3.2Resultados

Ganho de peso

O ganho de peso foi calculado pela diferença do peso do final e o do início do tratamento. A ANOVA de 2 vias demonstrou efeito do estresse [$F(1,23) = 6,903$; $p<0,05$], onde esses animais ganharam menos peso em relação aos controles. N = 4-5/grupo (Figura 3.1).

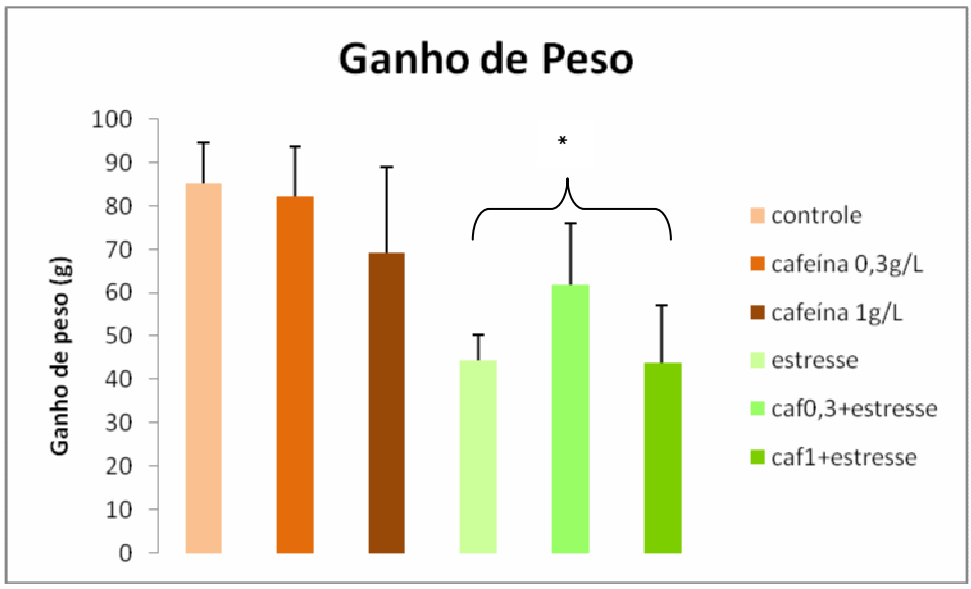

Figura 3.1 Ganho de Peso. Os dados estão expressos como média ± erro padrão. Efeito do estresse ($p<0,05$).

Exposição ao Froot Loops®

A ANOVA de medidas repetidas mostrou que todos os animais aumentaram o consumo [$F(4,59) = 43,854$, $p<0,001$] e diminuíram a latência [$F(4,59) = 30,408$, $p<0,001$] para comer os Froot Loops® nas sessões de treino (em restrição alimentar) ao longo dos dias. N = 6-13 (Figuras 3.2 e 3.3).

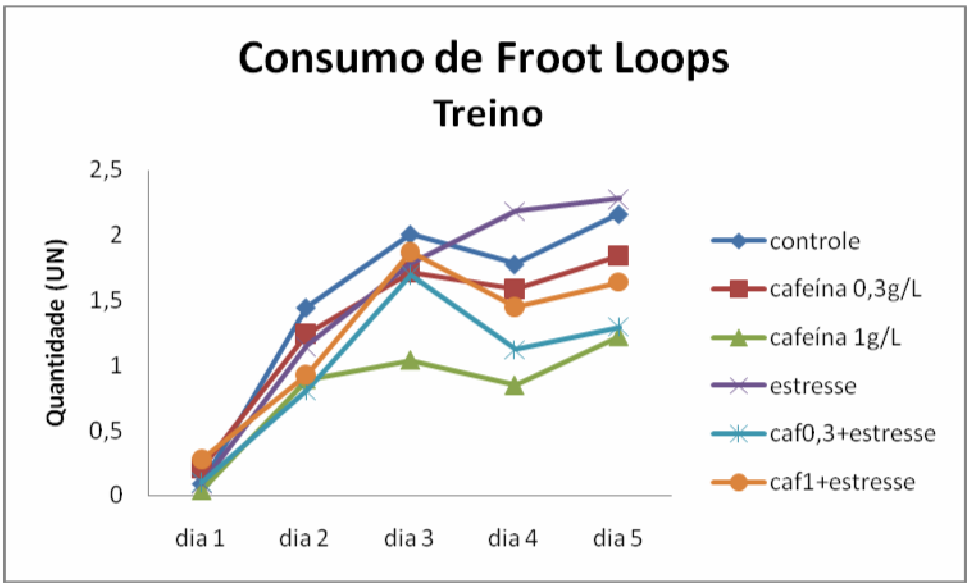

Figura 3.2 Consumo de Froot Loops® – Treino. Os dados estão expressos como média ± erro padrão. Efeito dos dias ($p<0,001$).

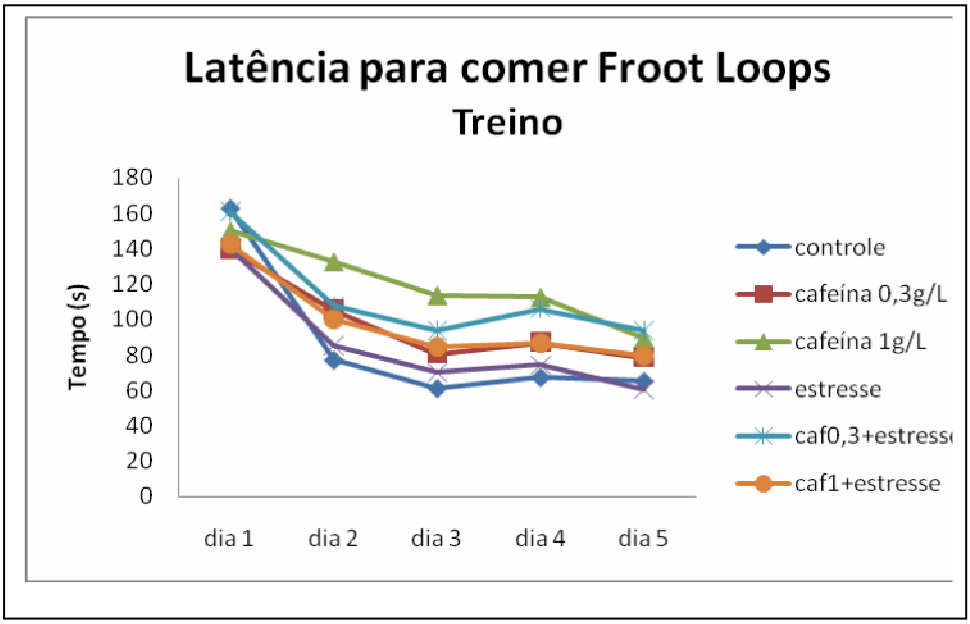

Figura 3.3 Latência para comer Froot Loops® – Treino. Os dados estão expressos como média ± erro padrão. Efeito dos dias ($p<0,001$).

Com relação à sessão de teste, a ANOVA de 2 vias demonstrou que os animais tratados com cafeína 0,3g/L apresentaram um aumento na latência para comer o alimento [$F(2,52) = 3,517$, $p<0,05$] enquanto que os animais tratados com cafeína 1g/L apresentaram um efeito marginal. Já no consumo de Froot Loops® no teste, tanto os animais tratados com cafeína 0,3g/L quanto com 1g/L apresentaram uma diminuição no consumo deste alimento [$F(2,52) = 4,241$, $p<0,05$]. Não houve efeito do estresse em nenhum destes parâmetros $p>0,05$. N = 6-13 (Figuras 3.4 e 3.5).

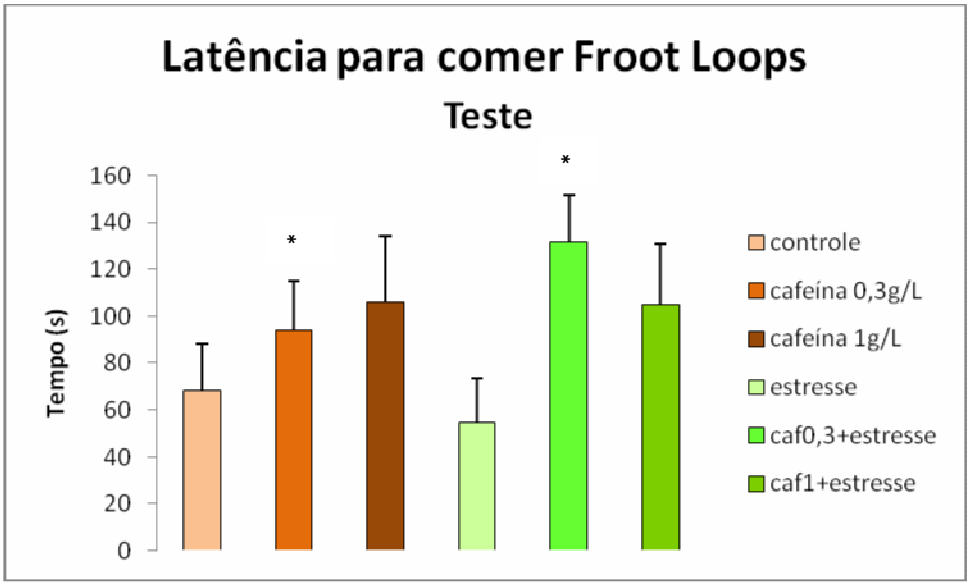

Figura 3.4 Latência para comer Froot Loops® – Teste. Os dados estão expressos como média ± erro padrão. Efeito da cafeína 0,3g/L ($p<0,05$).

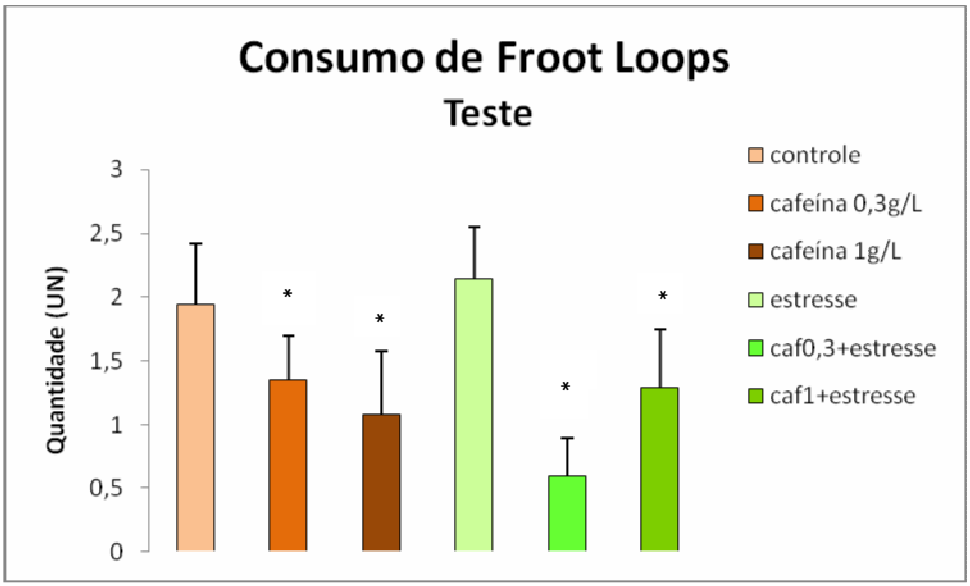

Figura 3.5 Consumo de Froot Loops® - Teste. Os dados estão expressos como média ± erro padrão. Efeito da cafeína 0,3 e 1g/L ($p<0,05$).

Exposição ao Cheetos®

A ANOVA de medidas repetidas mostrou que todos os animais apresentaram uma diminuição na latência para comer o Cheetos® ao longo dos dias de treino [$F(2,59) = 7,914$, $p<0,001$] (Figura 3.6). Com relação ao consumo de Cheetos® nas sessões de treino, os animais apresentaram um aumento no consumo ao longo dos dias [$F(2,59) = 42,844$, $p<0,001$]. Houve uma interação entre a cafeína e o tempo [$F(4,59) = 4,023$, $p<0,01$] onde a cafeína apresenta uma curva diferente com relação aos demais grupos ao longo dos dias, apresentando um aumento menos acentuado no consumo. Além disso, a cafeína 1g/L nos dias 2 e 3 de treino apresentou um consumo de Cheetos® menor com relação aos demais grupos [$F(2,59) = 4,247$, $p<0,05$]. N = 6-13 (Figura 3.7).

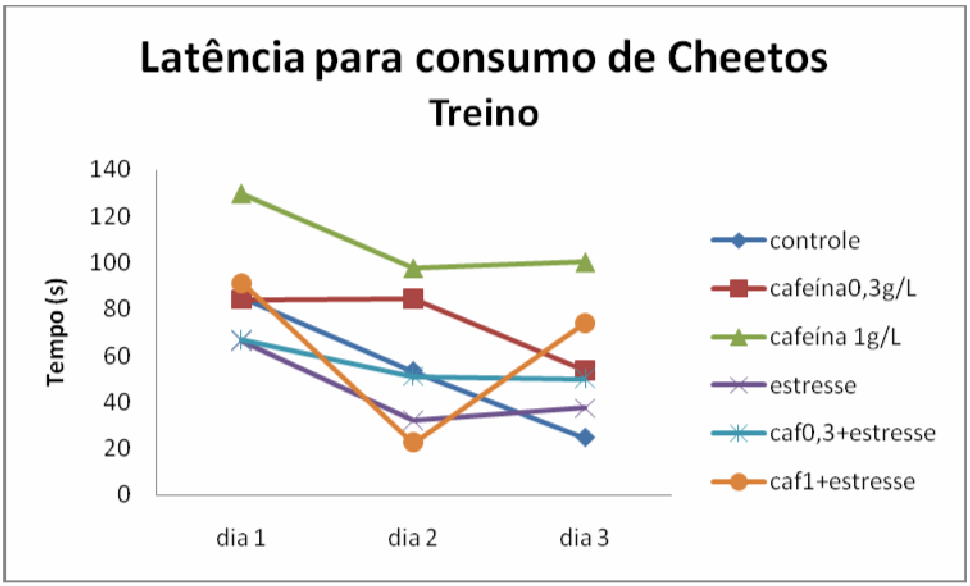

Figura 3.6 Latência para consumo de Cheetos® - Treino. Os dados estão expressos como média ± erro padrão. Efeito dos dias (p<0,001).

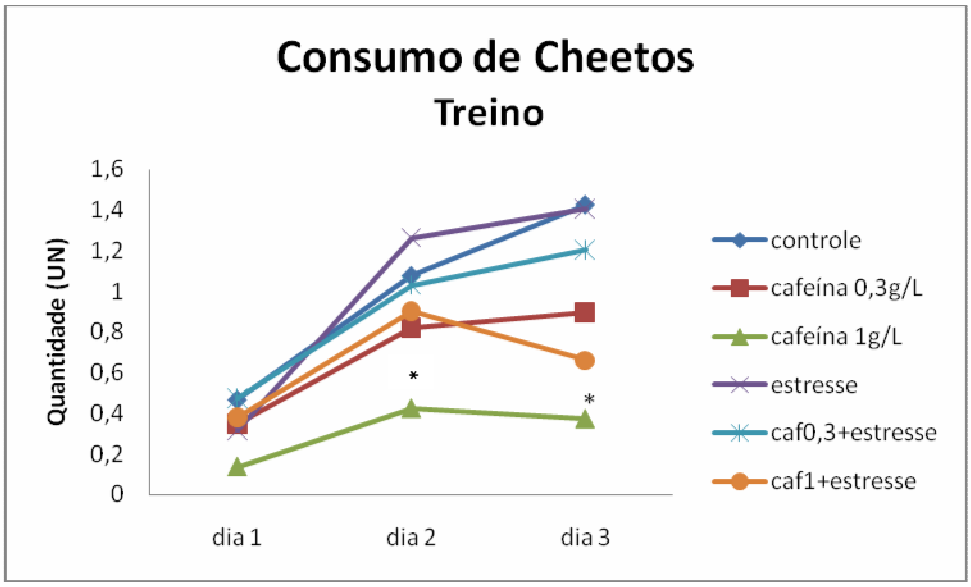

Figura 3.7 Consumo de Cheetos ® – Treino. Os dados estão expressos como média ± erro padrão. Efeito dos dias (p<0,001). Interação cafeína X tempo (p<0,01). Efeito da cafeína 1g/L nos dias 2 e 3 (p<0,05).

Com relação à sessão de teste não houve diferença entre os grupos na latência para comer o Cheetos® bem como no consumo deste alimento, $p>0,05$. N = 6-13 (dados não mostrados).

4. Discussão

Neste trabalho encontramos resultados relevantes como o aumento do comportamento do tipo ansioso pelo estresse crônico e pelo tratamento com a cafeína, o qual é sexo-específico, o aumento na latência para comer o Froot Loops® apresentado pelos grupos tratados com cafeína 0,3g/L e cafeína 1g/L(marginalmente), estressados ou não, bem como uma redução no consumo deste alimento por estes mesmos grupos. Além destes resultados comportamentais, essa dissertação apresenta resultados relacionados ao estresse oxidativo onde foi observado um aumento no dano ao ADN no hipocampo de ratos machos (efeito sexo-específico) tanto pelo estresse crônico quanto pelo consumo de cafeína, além de uma interação entre esses fatores. Com relação à atividade das enzimas antioxidantes, foram encontradas alterações dependentes da estrutura cerebral em questão. No estriado, o estresse aumentou a atividade da SOD e houve uma tendência a diminuir a atividade da CAT. No córtex, houve uma interação entre cafeína e estresse na atividade da SOD e da CAT, uma vez que a cafeína aumentou a atividade destas enzimas nos animais controles e não teve efeito nos estressados. Com relação ao TRAP, houve uma tendência a uma interação entre cafeína e estresse no hipocampo, pois a cafeína 0,3g/L aumentou o TRAP nos animais controle, enquanto nenhum efeito foi observado nos estressados.

Conforme descrito no Capítulo 1 não foi observado efeito na atividade locomotora no teste do Campo Aberto em machos e fêmeas, demonstrando o desenvolvimento de tolerância induzida pela ingestão crônica de cafeína, diferentemente do tratamento agudo com doses moderadas (ANTONIOU ET AL., 2005; KARCZ-KUBICHA ET AL., 2003) Por outro lado, o efeito da cafeína em ambas as doses, aumentando a ansiedade em ratos machos pode ser observado tanto no teste do Campo Aberto (pelo tempo gasto nos quadrados centrais do aparato) quanto no Labirinto em Cruz Elevado. Já o efeito do estresse no aumento da ansiedade só foi observado no teste do Labirinto

em Cruz Elevado. Este resultado, no entanto, está de acordo com um estudo onde foi demonstrado que o estresse por contenção pode induzir o comportamento do tipo ansioso no Labirinto em Cruz Elevado, sendo as alterações mais significantes em machos do que em fêmeas (CHAKRABORTI ET AL., 2007). Tem sido observado o envolvimento da adenosina em comportamentos relacionados à ansiedade. Sugere-se que a adenosina, agindo nos receptores A1, apresenta efeitos ansiolíticos e, agindo no receptor A2, provoca efeitos opostos (KULKARNI, SINGH e BISHNOI, 2007). Uma vez que a cafeína bloqueia os receptores de adenosina A1 e A2, mas induz efeitos ansiogênicos quando cronicamente administrada, podemos sugerir que este efeito pode estar relacionado ao antagonismo do receptor A1 (CORREA e FONT, 2008); no entanto, alterações adaptativas nos níveis dos receptores não devem ser descartadas (JOHANSSON ET AL., 1997).

A adenosina está envolvida na percepção do sabor doce. Foi observado que adaptações na língua humana às metilxantinas, em concentrações que inibem os receptores de adenosina, potencializam o sabor. Esse efeito foi observado com o adoçante artificial acesulfam-K (o qual tem um componente amargo), com NaCl e quinina e foram confirmados em estudos animais (SCHIFFMAN, GILL e DIAZ, 1985). Com base nestas observações, o aumento na latência e a diminuição no consumo de Froot Loops® pela cafeína 0,3g/L e 1g/L na sessão de teste, bem como o fato da cafeína 1g/L durante as 2 últimas sessões de habituação apresentar um consumo de Cheetos® de forma menos intensa com relação aos demais grupos, pode estar relacionado a uma alteração na percepção do sabor destes alimentos em virtude do tratamento crônico com cafeína. É interessante observar que, quando administrada de forma aguda, a cafeína não apresenta esse efeito (PETTENUZZO ET AL., comunicação pessoal). Vale ressaltar, que não houve efeito da cafeína no ganho de peso dos ratos submetidos ao

comportamento alimentar, provavelmente pelo fato de a exposição aos alimentos (Froot Loops® e Cheetos®) ter ocorrido por um breve período de tempo. É interessante observar que a ansiedade pode levar a um aumento no consumo de alimento doce (ELY ET AL., 1997) e, neste trabalho, embora os animais tratados com cafeína apresentem maior ansiedade, mostram um consumo reduzido de alimento doce, sugerindo outros mecanismos para esse efeito sobre o comportamento alimentar. Uma relação com a ansiedade não pode, porém, ser descartada, pois é possível que diferentes níveis de ansiedade levem a distintas ações sobre o comportamento.

Além de verificar os efeitos do consumo de cafeína e do estresse crônico sobre parâmetros comportamentais como a ansiedade e o comportamento alimentar, este trabalho também verificou os efeitos destes fatores sobre parâmetros bioquímicos relacionados ao estresse oxidativo em estruturas cerebrais. Para avaliar o dano oxidativo utilizamos duas técnicas, TBARS (para avaliar lipoperoxidação) e Cometa (para avaliar o dano ao ADN). O TBARS foi realizado no hipocampo, estriado e córtex de ratos machos e não foram observadas diferenças entre os grupos. Estes resultados concordam com alguns dados da literatura (MANOLI ET AL., 2000; ALPTEKIN ET AL., 1996). Vários estudos, no entanto, têm demonstrado aumento no TBARS em estruturas cerebrais de animais cronicamente estressados (FONTELLA ET AL., 2005; AKPINAR ET AL., 2007; DERIN ET AL., 2006). Estas diferenças podem estar relacionadas ao uso de diferentes modelos de estresse crônico (tipo de estressor e período de exposição ao estresse). A avaliação do dano ao ADN pela técnica do Cometa foi realizada em hipocampo de ratos machos e fêmeas. Nos machos, o consumo de cafeína e o estresse crônico aumentaram o dano ao ADN. A cafeína é um alcalóide e seus produtos de degradação, teobromina e xantina podem reduzir o cobre de Cu(II) a Cu(I), levando a geração de radicais do oxigênio *in vitro* (SHAMSI e HADI, 1995). A produção de

radicais do oxigênio, aumentando o estresse oxidativo, pode levar a quebras do ADN (AZAM ET AL., 2003). Entretanto, as quebras do ADN surgem a partir do dano oxidativo, mas também a partir do processo de reparo do ADN (HALLIWELL e WHITEMAN, 2004) e, dessa forma, o dano ao ADN observado nestes ratos não significa que seja um dano permanente. Além disso, houve uma tendência a uma interação entre estresse e cafeína no TRAP, pois a cafeína 0,3g/L aumentou o TRAP nos animais controle, enquanto nenhum efeito foi observado nos estressados. Isso sugere que a cafeína pode ter propriedades neuroprotetoras, mas esse efeito depende da dose e da condição do animal uma vez que esse efeito protetor não foi observado nos ratos cronicamente estressados. Tem sido demonstrado que a exposição ao estresse repetido induz o estresse oxidativo, especialmente no hipocampo (FONTELLA ET AL., 2005), onde a produção de EROs, como peróxidos, radical hidroxila e superóxido, podem levar ao dano oxidativo celular pela quebra do ADN (MUQBIL, AZMI e BANU, 2006). Neste trabalho, observamos que ratos estressados cronicamente apresentaram um aumento na relação SOD/CAT no hipocampo, contribuindo para a vulnerabilidade ao estresse oxidativo, uma vez que a SOD é a enzima responsável por converter o radical superóxido em peróxido de hidrogênio, e este, deve ser degradado pela ação da CAT e/ou da GPx para evitar a formação de radical hidroxila (o qual é altamente danoso) (HALIWELL, 2001). Baseado nisso, o aumento no dano ao ADN observado no hipocampo dos ratos estressados cronicamente pode estar relacionado ao aumento da relação SOD/CAT. Ainda com relação ao dano ao ADN, houve uma interação entre estresse e cafeína, pois quando esses dois fatores foram combinados, o efeito não foi maior do que os seus efeitos separados. Isso pode ser devido a um efeito teto ou uma ação inibitória da cafeína na resposta ao estresse, no entanto, essa última opção não é

apoiada pelos níveis de corticosterona plasmáticos, uma vez que o efeito inibitório da cafeína na resposta ao estresse foi observado apenas em fêmeas.

Em dois parâmetros, onde analisamos diferenças sexo-específicas (ansiedade e dano ao ADN) as fêmeas mostraram ser mais resistentes do que os machos aos efeitos da cafeína e do estresse. É importante lembrar que, com relação à avaliação do dano ao ADN em fêmeas, apenas um estudo piloto foi realizado e um aumento na amostra é necessário para uma melhor interpretação. Tem sido sugerido que hormônios gonadais, principalmente o estradiol, podem contribuir para que ratas adultas apresentem resistência ao estresse crônico (LUINE ET AL., 2007), uma vez que o estradiol pode agir como um agente ansiolítico (HILL, KARACABEYLI e GORZALKA, 2007).

Conforme descrito no Capítulo 2, o estresse crônico e o tratamento com a cafeína causam alterações na atividade das enzimas antioxidantes, que diferem dependendo da estrutura cerebral. No estriado, o estresse aumentou a atividade da SOD e houve uma tendência a diminuir a atividade da CAT. Esse resultado sugere um possível aumento na produção de EROs no estriado, uma vez que estas espécies químicas parecem aumentar diretamente a expressão da SOD (WARNER ET AL., 1996; YOO, CHANG e RHO, 1999).

No córtex, houve uma interação entre cafeína e estresse na atividade da SOD e da CAT, pois a cafeína aumentou a atividade destas enzimas nos animais controles e não teve efeito nos estressados. Isso sugere que a cafeína possa desempenhar um papel protetor nessa estrutura, mas apenas nos animais não-estressados. Além disso, houve uma diminuição na atividade da GPx nos animais estressados.

Segundo outros autores (GASIOR, 2000; SVENNINGSSON, NOMIKOS e FREDHOLM, 1999), os quais usaram doses semelhantes e mesma rota de administração de cafeína utilizadas neste estudo, as concentrações encontradas no

plasma estão próximas ao Kd da cafeína para receptores da adenosina. Portanto, os efeitos da cafeína encontrados neste estudo provavelmente são devido ao seu antagonismo aos receptores de adenosina. E, sendo que alguns efeitos da cafeína não foram observados nos animais submetidos ao estresse comportamental, é possível que o estresse esteja alterando o sistema adenosinérgico e/ou outros sistemas de neurotransmissores afetados pela adenosina no sentido de compensar os efeitos da cafeína. Uma posterior quantificação destes receptores nestes tratamentos é necessária para um melhor entendimento destes mecanismos.

5.Conclusões

- O estresse crônico e o consumo de cafeína levaram a um aumento no comportamento do tipo ansioso, o qual é sexo específico, sendo observado apenas em ratos machos.
- A cafeína, em ambas as doses, promoveu um aumento na latência e uma diminuição no consumo de Froot Loops®, em ratos previamente alimentados.
- O consumo de cafeína e o estresse crônico não induziram qualquer alteração na lipoperoxidação nas estruturas avaliadas.
- O estresse crônico e o consumo de cafeína promoveram um aumento no dano ao ADN em hipocampo de ratos, efeito que foi sexo específico. Além disso, houve uma interação entre os dois fatores, pois quando esses foram combinados, o efeito não foi maior do que os seus efeitos separados.
- O estresse repetido por contenção induziu um estado de maior susceptibilidade ao estresse oxidativo em algumas estruturas cerebrais devido a atividade alterada das enzimas antioxidantes.
- No hipocampo houve uma tendência a uma interação entre estresse e cafeína no TRAP, pois a cafeína 0,3g/L aumentou o TRAP nos animais controles, enquanto nenhum efeito foi observado nos estressados. Isso sugere uma ação neuroprotetora da cafeína. Por outro lado, nesta mesma estrutura, ratos estressados apresentaram um aumento na relação SOD/CAT sugerindo uma maior produção de radicais livres o que pode ter contribuido para o dano oxidativo do ADN.
- No estriado, o estresse aumentou a atividade da SOD e houve uma tendência a diminuir a atividade da CAT sem alterar a atividade da GPx.
- No córtex, houve uma interação entre cafeína e estresse na atividade da SOD e da CAT, uma vez que a cafeína aumentou a atividade destas enzimas nos

animais controles e não teve efeito nos estressados. Além disso, houve uma diminuição na atividade da GPx nos animais estressados.

- A administração crônica de cafeína levou em alguns casos (especificamente no córtex) a um aumento na atividade de enzimas antioxidantes, sugerindo um papel neuroprotetor, que depende do estado do sujeito (estressado ou não).

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